Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control

Updated and Revised Reprint

Edited by:

Eckardt Johanning M.D., M.Sc.

Aspergillus heads

mold in home

composting facility

home biogarbage container

Mount Sinai School of Medicine, New York, New York
Department of Community Medicine
Fungal Research Group, Inc. (FRG)

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Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control - Update and Revised Edition

Edited by Dr. med. Eckardt Johanning M.D., M.Sc.

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Contents

Introduction

Conference organizer 10
2nd Edition - Introduction 10
Introduction by Eckardt Johanning 12
Greetings by Joseph Forgacs 22
Greetings by H. Bruno Schiefer 24

Chapter 1
Health Effects; Pathology; Epidemiology

Effects after mold exposure - which are the causative agents? 28
Ragnar Rylander

Ecology, detection and identification problems of moulds in indoor environments 33
Robert A. Samson

Occupational exposure to molds; diseases and diagnosis 38
Kari Reijula

Indoor moulds: a public health problem in Belgium: overview of 15 years' experience 48
Nicole Nolard

Pilot analysis of the immune response to fungal antigens in subjects working in humidity damaged houses 54
Anja S. Tiilikainen, Irmeli Heikkinen, Kirsi Vedenpää, Riitta Karttunen,

Can microbial volatile metabolites cause irritation at indoor air concentrations? 60
Anna-Liisa Pasanen, Anne Korpi, Jukka Pekka Kasanen, Pertti Pasanen

Growth conditions of streptomyces anulatus regulate induced inflammatory responses and cyto toxicity in macrophages 66
Maija-Riitta Hirvonen, Merja Suutari, Ulla Lignell, Marjo Ruotsalainen, Aino Nevalainen

Clinical findings related to indoor fungal exposure - review of clinic data of a specialty clinic 70
Eckardt Johanning, Paul Landsbergis

Pulmonary hemorrhage among infants with exposure to toxigenic molds: an update 79
Ruth A. Etzel, Dorr G. Dearborn

IAQ and human toxicosis: empirical evidence and theory 84
Harriet M. Ammann

Cognitive impairment associated with exposure to toxigenic fungi 94

Wayne A. Gordon, Eckardt Johanning, Lisa Haddad

Symptoms associated to work in a water damaged school building 99
T. Sigsgaard, H.L.C. Jensen, E. Nichum, S. Gravesen, L. Larsen, M.Ø. Hansen

Sensory irritation of microbially produced volatile organic compounds in mice during repeated exposures 106
Anne Korpi, Jukka-Pekka Kasanen, Anna-Liisa Pasanen

Immunological biomonitoring in the assessment of exposure to airborne fungi from waste handling 112
J. Bünger, M. Müller, K. Stalder, E. Hallier.

Waste handling is strongly associated with dyspnoea 119
Niels Ebbehøj, Niels O. Breum, Gerard J. Coenen, Ulla I. Ivens, Birgitte Kjær, Thomas Lund, Poul Suadicani, Uffe Midtgaard, Helle Würtz, Torben Sigsgaard

An assessment of exposure and respiratory health among sewage treatment pressroom workers 125
Michael B. Lax, Greg Siwinski, Federica A. Manetti

Chronic toxic encephalopathies apparently related to exposure to toxigenic fungi 131
Pierre L. Auger, Pierrot Pépin, J. David Miller, Manfred Gareis, Julien Doyon, Rémi Bouchard, Marie-France Pinard, Claude Mainville

Inhalation of (1→3)-β-D-glucan in humans 139
L. Beijer, J. Thorn, R. Rylander

Building-related illness in occupants of mold-contaminated houses: A case series
James Craner 146

Diagnosing the cause of a "sick building:" a case study for an epidemiological and microbiological investigation 158
James Craner, Linda D. Stetzenbach

Fungal exposure and IgG-levels of occupants in houses with and without mold problems 166
A. Hyvärinen, M. Reiman, T. Meklin, T. Husman, M. Vahteristo, A. Nevalainen

The immunopathology of hypersensitivity reactions 169
Vincent A. Marinkovich

Exposure to *Stachybotrys chartarum* induces immunoglobulin A antibody response in man 174
Päivi Raunio, Anna-Liisa Pasanen, Tuula Husman, Tuomas Virtanen

Impacts of airborne viruses on indoor environments 179
Douglas T. Reindl

Sensitization to molds and respiratory symptoms in school children 186
T. Taskinen, A. Hyvärinen, T. Meklin, T. Husman, A. Nevalainen, M. Korppi

Glucan exposure and airways inflammation among household waste collectors in two municipalities in Sweden 190
Jörgen Thorn, Lena Beijer, Ragnar Rylander

Moisture observations and health 195
Mikko Vahteristo, Jouni Räsänen, Tuula Husman, Aino Nevalainen

Chapter 2

Exposure Assessment; Analytical Methods

Mycotoxin cytotoxicity screening of field samples 202
Manfred Gareis, Eckardt Johanning, Ritchie Dietrich

The effect of inhaled spores of mycotoxin producing fungi on animals 214
Eeva-Liisa Hintikka

Trichothecenes as a potent inducer of apoptosis 221
Hiroki Okumurai, Naoto Yoshino, Yoshitsugu Sugiura, Masao Sugamata, Eeva-Liisa Hintikka, Bruce Jarvis, Yoshio Ueno

Analysis for *Stachybotrys* toxins 232
Bruce B. Jarvis, Simon F. Hinkley

Assessing bioaerosols in elementary school classrooms 240
K.H. Bartlett, S.M. Kennedy, M. Brauer, B. Dill, C. vanNetten

Anatomy of a fungal problem 245
Neil Carlson, Arif Quraishi

Characterization of bioaerosol emissions from a suburban yard waste composting facility 254
Luke Curtis, Mary Ross, Joe Chung, Peter Scheff, Victoria Persky, Richard Wadden, Viswanathan Ramakrishnan, Daniel Hryhorczuk

Airborne contamination of Eastern Canada sawmills 258
Caroline Duchaine, Anne Mériaux, Yvon Cormier

Prevalence of fungi in carpet dust samples 268
Mark Hodgson, Richard Scott

Detection and decontamination of a facility contaminated with fungi including *Stachybotrys chartarum*: a case study 275
Ling-Ling Hung

Case study: Airborne concentrations of *Trichoderma* and *Stachybotrys* linked to mycotoxicosis 282
Larry D. Robertson

Fungal genera identified from flooded wall cavities 287
Constance L. Jenkins, Peter H. Sierck

Chapter 3

Microbiology

Exposure measures for studies of mold and dampness and respiratory health 298
J. David Miller, Robert Dales, Jim White

Fungal growth in buildings: the aerobiological perspective 306
Harriet A. Burge

Why are there still problems with fungal allergen extracts? 313
W. Elliott Horner, Samuel B. Lehrer

From fungal exposure to disease: a biological monitoring conundrum 320
Raymond E. Biagini

Comparative studies of fungal media for the recovery of *Stachybotrys chartarum* from environmental samples 330
Stella M. Tsai, C. S. Yang, P. Heinsohn

Heteroduplex DNA fingerprinting of *Penicillium brevicompactum* from house dust 335
James A. Scott, Neil A. Straus, Bess Wong

The Trichodiene Synthase Gene from *Stachybotrys chartarum*: A potential diagnostic indicator of indoor contamination 343
Neil A. Straus, James Scott, Bess Wong

Microscopic fungi and their metabolites in dwellings - a bioassay study 351
Elena Piecková, Zdenka Jesenská, Ken Wilikins

Moisture, mold and health in apartment homes 355
A. Nevalainen, M. Vahteristo, J. Koivisto, T. Meklin, A. Hyvärinen, J. Keski-Karhu, T. Husman

Toxigenic microbes in indoor environment: identification, structure and biological effects of the aerosolizing toxins 359
M.S. Salkinoja-Salonen, M.A. Andersson, R. Mikkola, A. Paananen, J. Peltola, H. Mussalo-Rauhamaa, R. Vuorio, N-E. Saris, P. Grigorjev, J. Helin, U. Koljalg, T. Timonen

An assessment of the occurrence of mould bioaerosols in the in- and outdoor environment of shacks in Durban, South Africa 375
N. Gqaleni, S. Danaviah, S. Bux, AA.Chuturgoon, M.F. Dutton

Evaluation of exposure to environmental bacteria 381
Laitinen Sirpa, Kangas Juhani

Analysis of microbial contamination of a ventilation system detected by measurement of microbial volatile organic compounds 386
Marion J. Fedoruk, Steven Uhlman, Dean B. Baker, Haiou Yang

Mycobacteria and related genera are major colonizers of a wall in a children's day care center 396
Maria Andersson, Irina Tsitko, Riitta Vuorio, Mirja S. Salkinoja-Salonen

Cellular and humoral responses in an animal model inhaling *penicillium* chrysogenum spores 403
J. Danny Cooley, Wing C. Wong, Cynthia A. Jumper, David C. Straus

Sporulation of the hyphomycete *Stachybotrys chartarum* under three light conditions 411
Patricia Heinsohn, Sharon Harney, K. Alexandros Exuzides

Mycotoxin spectra as a biochemical parameter for occupational and environmental

fungus exposure 418
M. Müller, J. Bünger, E. Hallier

Production of mycotoxins on water damaged building materials. 423
Kristian Fog Nielsen, Suzanne Gravesen

Membrane toxic substances in water-damaged construction materials and fungal pure cultures. 432
Joanna Peltola, Maria M. Andersson, Raimo Mikkola, Helena Mussalo-Rauhamaa, Mirja S. Salkinoja-Salonen

Effects of *Stachybotrys chartarum* spores and toxin on alveolar surfactant phospholipid composition and concentration in mice 444
M.W. Sumarah, T.G. Rand, M. Oulton, C.D. Mason, J. MacDonald, M. Anthes

Different methods to characterize moldy buildings 453
Mika Toivola, Marjut Reiman, Anne Hyvärinen, Teija Meklin, Aino Nevalainen

Comparative studies of collection efficiency of airborne fungal matter using Andersen single-stage sampler and Air-O-Cell cassettes 457
Stella M. Tsai, Chin S. Yang, Patrick Moffett, Andrew Puccetti

Trichothecene mycotoxins in some water-damaged buildings 465
Tapani Tuomi, Lauri Saarinen, Sanna Lappalainen, Outi Lindroos, Marjo Nikulin, Kari Reijula

Chapter 4
Applied Research

Sarcoidosis and exposure to occupational and environmental agents. 476
Carol Ortiz, Michael Hodgson, Daniel McNally, Eileen Storey

Immunochemical detection of mycotoxins associated with Stachybotryotoxicosis 482
R. Dietrich, E. Johanning, M. Gareis, E. Schneider, E. Usleber, E. Märtlbauer

Chapter 5
Prevention, Mitigation and Control

Mitigation of visible fungal contamination in buildings: Experience from 1993 - 1998 494
Philip R. Morey, Daryl Sawyer

Microfungal contamination of damp buildings: biological aspects. 505
Suzanne Gravesen

During removal of bioaerosol contaminated roof substrate material in an occupied facility 512
William A. Turner, Steve M. Caulfield, Francis A. Patnode, Gerard Blanchette, Terry Brennan

Characterization, prevention, and control of mildew in residential environments: an applied research study 516
Eugene C. Cole, Carl E. Cook, Pamela D. Dulaney, Keith E. Leese, Michael S. Newell, Mehboob U. Ahmed, Laurie Selman, Holly A. Bowers, April L. Corbett, Cari J. Cole

ACGIH TLV Statement on bioaerosols; American Council of Government Industrial Hygienists 520
Harriet M. Ammann

Industrial hygiene & clearance considerations for a microbial remediation project 523
John A. Tiffany, Howard A. Bader, Adam J. Pratt

Microbes and moisture content of materials from damaged building 529
T. Meklin, S. Haatainen, E. Kauriinvaha, A.-V. Kettunen, U. Haverinen, M. Vahteristo, M. Viljanen, A.Nevalainen

Use of MVOC measurements and odor perception as indicator of mould in indoor areas 532
R. Keller, K. Senkpiel, H. Ohgke

A model for assessing health risks associated with exposures to legionella bacteria..... 538
Brenda E. Barry, Jerry F. Ludwig, John F. McCarthy

Concentrations of viable spores of fungi and actinomycetes in ventilation channels ... 541
Outi Lindroos, Sanna Lappalainen, Kari Reijula

Fungi and endotoxin exposure in paper and glass recycling plants 544
H. Würtz, N.O. Breum, N. Ebbehøj, O. Jorgensen, U. Midtgaard, N. Nielsen

Air quality restoration in a fungal contaminated building; a case study 549
Herman Sabath

Sampling, results & remediation in 300 "sick houses" 554
Jeffrey C. May

Identifying and preventing fungal contamination problems in new home construction 561
Päivi Salo

A toxic mold cleanup guide 568
Jim H. White

Chapter 6
Public and Occupational Health Issues

Development of a Public Health Intervention Program in Finland..... 574
T. Husman, A. Nevalainen

An assessment of mold contamination problems in Atlantic Canada schools:mold burdens, amplifying sites and benefits of proactive school inspection policies..... 581

Special Section

Molds as an environmental factor in infant leukemia? 594
L. Casteleyn, K. Van Damme, H. Van den Berghe

Microbial contamination litigation: expert witnesses and scientific evidence 595
Edward H. Cross

Field report: Initial microbiological assessment in four new condominiums 603
Michael McGuinness, Patrick D. McGuinness

Learning from *Stachybotrys Chartarum*: How to find hidden mold in buildings 611
Claude Mainville, Marie-France Pinard, Louis Gagnon, Robert Kelley, Alain Beaudet

Concentrations of airborne viable spores of fungi and actinomycetes in mold-damaged offices and schools 616
Sanna Lappalainen, Outi Lindross, Kari Reijula

Allergens, diagnosis, and pathogenesis 620
Viswanath P. Kurup

Up-date 627

Exposure assessment and health effects - a follow-up meeting to the 3rd International Conference on Fungi, Mycotoxins and Bioaerosols (Saratoga Springs, N.Y.) 628
Aino Nevalainen, Eckardt Johanning, Tjina Reponen, Kari Reijula, Tuula Husman

Guidelines on assessment and remediation of fungi in indoor environments 635
New York City Department of Health Bureau of Environmental & Occupational Disease Epidemiology

Web-sites recommended by various authors for general information and as resources . . 652

Photos 655

Authors 657

Index 661

Color Plates 671

These are the proceedings of the

THIRD INTERNATIONAL CONFERENCE ON FUNGI, MYCOTOXINS AND BIOAEROSOLS

Health Effects, Assessment, Prevention and Control.

September 23-25, 1998 · Saratoga Springs, New York, U.S.A.

CONFERENCE ORGANIZER

This conference was organized by the Eastern New York Occupational and Environmental Health Center (ENYOEHC), one of eight New York State clinical centers of excellence, providing diagnostic, treatment and prevention services for occupational and environmental diseases. This clinic network was created by the New York State Legislature in 1987. ENYOEHC is administered by Kaiser Permanente, Northeast Division, and serves 22 counties along the eastern corridor of New York State.

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The editor.

2ND EDITION - INTRODUCTION

This is the 2nd edition with some minor corrections and updates requested by a few authors. It contains a summary of the "current state of knowledge" formulated at the recent Bioaerosols, Fungi and Mycotoxins satellite meeting in Espoo, Finland, which was held in connection with the Healthy Buildings 2000 conference, August 6-10, 2000. Further, the revised "New York City Department of Health Mold Remediation Guidelines" have now been released and are included for public distribution. It incorporates the scientific and special interest positions and tries to present a practical approach and solutions to indoor mold contamination, assessment, remediation and control. Indoor mold contamination and its public health impact have continued to gain further importance and scientific research interest. Advanced sampling technology and non-allergic health implications, i.e., toxic and inflammatory effects on the nervous, endocrine

and reproductive systems are receiving more attention. New research results will help to understand the complexity of fungal exposures, individual susceptibility and indoor fungal diseases. We included WEB Sites that will keep the reader better informed about this work and the results of the various international workgroups.

For information about the next meeting scheduled for the fall of 2002, please check: www.fungalresearchgroup.com

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INTRODUCTION BY ECKARDT JOHANNING, M.D., M.SC. CONFERENCE DIRECTOR

FUNGI IN INDOOR ENVIRONMENTS - A CHALLENGE FOR SCIENTIFIC RESEARCH AND PUBLIC HEALTH

This book contains the proceedings of the *Third International Conference on Fungi, Mycotoxins and Bioaerosols - Health effects, Assessment, Prevention and Control*, a scientific meeting that brought together physicians, veterinarians, industrial hygienists, mycologists, chemists, public health specialists, toxicologists, and laboratory specialists among many others. Four years have passed since our first International Conference in Saratoga Springs, New York: *Fungi and Bacteria in Indoor Air Environments* in fall of 1994 (Johanning and Yang, 1995). Again, we gathered a wide range of health professionals, indoor air consultants, research scientists and policy makers from universities and governmental agencies, such as Environmental Protection Agency (EPA), National Institute for Occupational and Environmental Health (NIOSH) and the Department of Health (DOH) - and last but not least the stake holders: representatives of patients, insurance companies, building managers, lawyers and unions to present new research results and to discuss the health implications of microbials in indoor environments or industries. Following our second meeting in 1996 that focused more on specific technical issues related to microbial remediation in buildings, we felt it was again time to get together, to talk and to compare notes about this theme that has become such a "hot topic" for the scientific community, but also for the public and the media. It became truly an international meeting with almost 350 people, and about half of the participants coming from around the world, from Belgium, Canada, Denmark, Finland, Germany, Japan, Netherlands, Norway, Slovakia, South Africa, Sweden and other countries - with more than 90 original presentations on the health effects, assessment, prevention and control of bioaerosols, fungi and mycotoxins at home or in the workplace. The stated learning objectives were to

- Discuss current data and new information on exposure, assessment, analytical methods, applied research, pathology, epidemiology, microbiology, health effects, prevention and mitigation of bioaerosol exposure
- Recognize the importance of bioaerosol exposure and adverse health effects
- Develop a multi-disciplinary team approach for the recognition and management of bioaerosol exposure.

This meeting has become recognized as a unique forum to present original research to a critical peer-audience, to discuss public health policies, but also to review concrete technical issues about detection and control of fungi and bacteria in our environment. The presented papers and scientific debate also showed that this is a topic of international concern. It crosses national barriers and affects different climactic zones. Since our first meeting in 1994 there is hardly any professional conference meeting on indoor air quality and health issues that does not mention moisture and water damage-related mold problems in buildings. The apparent widespread nature of the problems and the far reaching public health implications are gaining wider recog-

nition among professionals and the lay public, but surely not without controversies and conflict of interest in some cases.

THE SCOPE AND THE NUMBERS

The 'ingredients' for problems with unusual microbial exposure inside buildings appear readily available in many places and affect a wide range of people: water and organic building materials (cellulose). In other words: Moisture and condensation problems, water pipe leaks, flooding and storm-related defects, occurring in buildings and homes with wood, paper sheeted gypsum board or fiberglass insulation, carpets and furniture can result in significant microbial contamination if the water intrusion is neglected. Furthermore, many old and new industries are exposing workers to fine organic dust (microbial materials that become airborne) due to increased mechanized processing or handling of recyclable organic materials and waste that should not end up in landfills. A wide range of workers in various industries is potentially at risk for high exposure to microbials (fungi, bacteria, viruses) or its products (allergens, endotoxins, glucans, mycotoxins).

Number of workers in different industries with potentially significant microbial exposures:

Industry:	No. of workers with "organic dust" exposure:
• Farming	1.3 Mill.
• Lumbar/Wood	818000
• Food	1.7 Mill.
• Textile mills	590000
• Paper production	682000
• Agricultural service	786000
• Museum, Botanical and Zoological gardens	98000
• Garden/Building material	1 Mill.

A number of health problems related to moisture defects in buildings and occupational exposure to organic type of dust has been clinically observed. These have been coined a new challenge to occupational medicine (Reijula 1996. Johanning et al. 1998). Nevertheless, the inhalation of organic dust, defined as dust of vegetable, animal, or microbial origin, and related diseases has been well recognized and described earlier. However, it was thought to be mainly related to agricultural and farming activities (cotton processing, handling of animals, grain and food. But now we begin to recognize that also in non-agricultural environments significant exposure may occur accidentally. Two publications have well documented some of these earlier findings by various investigators in the agricultural and indoor environment (Rylander and Jacobs, 1994; Samson et al. 1994).

We are learning now that excessive exposure to such 'organic dust' can principally also occur in indoor environments and that chronic moisture problems may be very common in many

buildings. Most of us spend the greater part of our lives inside buildings and vehicles - and depend more often on mechanical ventilation for "fresh air", heat, cold and moisture. The emphasis on the reduction of building costs and maintenance, and the need for energy conservation has resulted in "tighter" buildings that appear to be more prone to aging-related defects and system failures. In our experience, all too often, current building technology and management do not satisfy our needs for "good air quality", free of harmful pollution or even for general comfort. The reason for most ventilation failures and problems are often not complicated and easily correctable, as EPA and NIOSH studies have shown. "Sick building"-related health complaints by our patients are very common, in particular in the occupational and environmental health clinics. Some medical sociologists suggest that indoor air quality (IAQ)-related complaints and diseases will be with us long into the next millennium and will gain greater importance than traditional chemical poisoning of industrial workers. Although our understanding of the multiple factors and their effects on air quality has increased considerably in the last decades there still remains a number of unanswered questions and scientific puzzles. Nevertheless, many of us IAQ experts believe, that if what we know now about tobacco smoke, volatile organic compounds, fibers, HVAC-systems, air pollution, etc. were applied and already-existing recommendations and standards were followed by the responsible parties, then most of the IAQ complaints would be resolved and the need for medical intervention would be substantially reduced.

However, there is still quite a task ahead of us and the numbers are impressive. In the U.S., of the total employed population of 131.2 Mill., approximately 70 Mill. people work indoors. In 1989 there were about 4.5 Mill. commercial buildings in the U.S. (28% service/shops, 15% offices, 14% assembly warehouses, 5% food services, 3% lodging and 2% food sales/health services). The Occupational Safety and Health Administration (OSHA) reported in the 1994 Indoor Air Quality (IAQ) proposal for a regulatory standard, that 30% of the 70 Mill. "indoor" workers have poor air quality (Federal Register, 1994). The National Institute for Occupational Safety and Health (NIOSH) reported that based on their review of previous health hazard evaluations (HETA) related to building/facility problems 42% of 104 buildings investigated had evidence of "water damage" (Crandall et al. 1996).

How healthy are our school buildings?

Our children and teachers spend a considerable portion of their life inside school buildings that are aging and often in need of repair. A recent governmental investigation revealed that of the more than 80000 schools in U.S., one third of them is in need of extensive repairs costing about 112 billion (GAO/HE'S, 1995):

- 14 Mill. students affected
- 50% of the surveyed schools reported „unsatisfactory environmental conditions:

Problem:	No. of schools	No. of students
Ventilation	21100	11.6 Mill.
Heating	15000	7.8 Mill.
Indoor air quality	15000	8.4 Mill.

Many schools and colleges have water leaks and mold problems, which are now increasingly monitored by local and state health departments, teachers' unions, i.e., the American Federation of Teachers (Washington, D.C.) and parents' organizations.

What about the indoor environmental quality in private homes in the U.S.?

Here are the statistics: There are 94,7 Million households or units in U.S. for the U.S. population of 270,5 Mill.:

- 61 Mill. owner occupied
- 33 Mill. renter occupied
- 59 Mill. single structures.

Of these homes, 47 Mill. were built between 1959 and 1975. The median age of the homes is 30-40 years. According to a U.S. government statistic, many of the buildings have leakage problems. Water leakage during last 12 months(U.S. Census, 1998 (most recent available statistics)):

- 10.9 Mill. (13%) U.S. homes had leakage from inside
  - 3.8 Mill. (34%) Fixture backups/overflow
  - 5.1 Mill. (46%) Pipe leaks

- 16.9 Mill. (21%) U.S. homes had leakage from outside
  - 7.1 Mill. (42%) roof
  - 6.1 Mill. (36%) basements
  - 3.0 Mill. (18%) walls.

In this context, it may come as no surprise that of the 9.7 Mill. owner-occupied units surveyed the most frequent repair within the prior two year period was: Roof repair (!), followed by building of additions, kitchen or bath remodeling/adding, siding replacement, etc. What does that tell us about the status and quality of the U.S. housing stock and the possibility of water intrusion and subsequent microbial growth?

Another source of "moisture" which is an important factor in indoor mold growth is heavy rains and flooding. During the spring of 1993, with the now called Great Mississippi Flood, flooding of 11,000,000 acres of land occurred - this is about one quarter of land area of the continental United States (see also: www.earthsat.com). Since then more widespread and recurrent flooding has occurred in many different parts of the U.S., often affecting the housing and causing substantial damage. A rapid cleanup of water-damaged homes can help to minimize widespread microbial problems, but special precautions and adequate personal protective equipment are recommended by official agencies (FEMA, 1997). It appears that a considerable number of children and adults in these are affected by allergy, asthma and other disorders as a result of it. The true impact of these natural disasters and mold problems on the public health

in these areas has not been systematically studied, although this would be an important project for prevention.

FUNGI AND HEALTH

The possible pathological consequences in humans and animals of exposure to bacteria and fungi fall principally into the categories of infection, allergy, and toxicity (Husman, 1996, Johanning 1999). In clinical practice the overlap of these pathological reactions of fungi is quite common. This may explain the wide variety of medical presentations and findings and why so many different medical specialties are involved in the care of such patients. Many medical providers, however, still have very little knowledge and training about air contamination problems and building-related diseases. This leads to delay in proper diagnosis and treatment failure, because the underlying causes are not recognized or evident problems with the IAQ (or now redefined as: Indoor environmental quality (IEQ)) are not corrected. Surely, a healthy individual with a good functioning immune system and respiratory organs should be able to handle most temporary adverse situations with IEQ problems, without any (lasting) harm and subsequent physical impairment. Many physicians are familiar with the concept of allergy and infection caused by fungal exposure, but irritative and toxic effects caused by fungi (or gram negative bacteria) in the form of inhalation of microbial by-products such as endotoxins, glucans and mycotoxins are still not widely known.

Mycotoxin-related diseases, life stock illnesses and death, and economic losses are very familiar to veterinarians and food safety specialists. References to the effects of a wide range of mycotoxins and other fungal-by-products can be found in medical sub-specialty fields. In the past, mycotoxins, which are naturally occurring chemical metabolites of fungi, have been implicated in cases of food poisoning, but research has shown that inhalation of mycotoxin-loaded spores and mycilia may have even greater potency and may be of concern for the individual with intense or prolonged exposures. Some of the now known and studied mycotoxins (there are several hundred different types) have been shown to have biological effects in humans and animals with various degrees of acute and chronic toxicity, cytotoxity, neurotoxicity, immunosuppressive effects, teratogenicity, mutagenicity, carcinogenicity, anti-tumor effects, and adverse endocrinologic effects. But much of this research has been done under laboratory conditions and little is still known about its impact in indoor environments and under "real-life" conditions when several fungal agents are present. However, past medical research has shown, that certain mycotoxins are able to target and adversely affect many body organs or systems, such as the lungs, skin, mucous membranes, the central and peripheral nervous system, the immune system (white blood cells), the liver, and the cardiovascular system and consequently cause a wide range of symptoms. On a molecular level they can interact and interfere with the DNA and RNA replication, transcription and translation, causing protein synthesis defects and inhibition, cell membrane defects and energy metabolism errors (Betina, 1989; Sharma RP and Salunkhe DK, 1991). Because of the unique potent effects of a few mycotoxins in humans and animals, they have been studied in many applications, from cancer chemotherapy to biological warfare research. Mycotoxins are considered important environmental pollutants. Small amounts of these compounds may result in serious health hazards, i.e., intoxication, cancer and

death (WHO, 1979, IARC, 1993), although in the past the ingestion route has been thought to be of greater importance in public health and food safety. More recent clinical-epidemiological cluster investigations of unusual disease outbreaks in homes, office buildings, court houses, and in the Cleveland area of lethal hemorrhagic lung diseases in infants have focused the attention on inhalation-related health risks of toxic fungi, such as *Stachybotrys chartarum* (a.k.a. *atra*), which may be more common and important in many cases of so-called "sick-building syndrome" or certain building-related diseases (i.e., bronchitis, pneumonitis)(Auger et al. 1994; Johanning et al. 1996; Hodgson et al. 1998; Etzel et al. 1998, Johanning, 1998). Such problems and outbreaks have not been sufficiently studied and documented in the past due in part to complex methodological and technical problems. From a statistical perspective research involving several exposure agents and disease outcomes occurring in small populations is very difficult and involves many limitations.

THE ALLERGY AND ASTHMA 'EPIDEMIC'

Nevertheless, several diseases of great public health importance are known to be either strongly associated, caused or substantially aggravated by exposure to fungi and microbial by-products, although the exact pathological mechanisms are still under investigation and often difficult to prove. One of these diseases is asthma, or more specifically occupational or environmental type asthma (Peat et al. 1998). In the USA there are about 12 Million people diagnosed with asthma. The numbers of recorded cases are steadily rising, with a jump of 29% between 1980 and 1987. There was also an increase in asthma-related deaths from 2000 per year reported for 1979 to 9394 in 1993. Besides the associated suffering in patients, the costs are enormous with an estimated $ 6.5 billion/year (medical expenses and indirect costs). It is estimated that about 40% may be saved with improved diagnosis and treatment, which also means better recognition of causes and exposure prevention. Occupational and environmental asthma is now the most common work-related respiratory disorder (2% to 15% of all adult-onset asthma). There are more than 450 substances in the workplace known to be associated with occupational asthma. Many of these are of biological origin, including fungi.

What about rhinitis and sinusitis, and plain allergy? Are these just "trivial" conditions? If you are not yourself a "sufferer"- just look at the numbers to get an appreciation of the scale of the problems. The number of people with allergic conditions is generally on the rise and more and more of these patients take some form of allergy medication. It is not certain, whether this may be a real trend or just a reflection of improved diagnostic tests and more reporting.

Allergic rhinitis statistic:

- 30 to 40 Mill. people in U.S.
- 19 Mill. employed adults
- 3.5 Mill. lost workdays annually
- 2 Mill. lost school days annually
- Direct costs: $2.4 billion

Sinusitis statistic:

- 35 Mill. (15%) people in U.S.
- 25% and more are related to allergy

Allergic rhinitis, sinusitis and asthma

- 20 80% of patients have a combined diagnosis.

Clearly, many cases of respiratory diseases, allergy and asthma are probably of multi-factorial origin and many substances have been implicated as causes in addition to genetic factors and host susceptibility. But what is important in this context here, is that public health research suggests that many of these diseases and exposure conditions could principally be prevented. There is wide agreement among the experts that it is prudent to avoid and minimize unnecessary exposure to fungi or bacteria. In particular exposure to known infectious, allergenic, toxic and carcinogenic fungi (i.e., *Aspergillus fumigatus, A. flavus, A. versicolor, Stachybotrys chartarum* and possibly some other fungal species) should not be tolerated because of what we know about their possible deleterious effects on animals and humans - even if we still do not know exactly, when, how and why people get sick from inhalation (or skin contact) of these fungal species (see also the recent publication of a related workshop with the topic: Indoor mold and children's health (Rylander and Etzel, 1999)).

In analogy to John Snow (a general practitioner in London, 1813-1858), who found out through pioneering simple epidemiological studies that the contaminated water made the people sick and advised to avoid certain water fountains in London, without initially knowing that it was the cholera microbe that caused the disease, it appears timely and prudent that public health advocates and agencies advise the public to follow the prudent hygiene principle: Do not live or work inside buildings with moisture and mold problems. Historically, this simple but "life saving" advice may even be traced back to the final of the 10 plaques and the Egyptian exodus, as well as to writings in the bible and other religious scripts, as some scholarly historians say (The Sunday Times, Aug. 16th, 1998). Indeed the fungal "poisons of the past" are thought to be the cause of the black plaque, the Salem witch-hunts, the "holy fire" (bizarre behavior caused by ergotism) and there appears to be good evidence to suggest that fungal food poisoning with mycotoxins has been responsible for the reduced fertility and the high mortality in the past centuries in Europe and Russia, during a time when large populations relied heavily on food (bread) contaminated with mycotoxins (Matossian, 1989). Better nutrition and generally improved hygiene have resulted in a resolution of these problems and substantial gains in the public health status.

Improving the indoor air and building quality cannot be solved by medical means alone, but requires social, political and economical attention. Although the results of recent research activities and field experiences presented in this book have given us much greater insight into this scientific topic and the understanding of fungal and bacterial disease is improving, there are still many unanswered questions and a lot of work remains to be done. This will require continued research efforts and learning from field and laboratory studies, and dissemination of information in publications, professional meetings and continuous medical/professional education. Most of all, we will have to work as multi-disciplinary teams and independent studies

with appropriate funding need to be conducted. The past conferences and this book are a demonstrated concerted effort to move in this direction.

As we said earlier in the proceedings of the first international meeting, the lay reader should be reminded to consider, that fungi and bacteria are ubiquitous and indeed part of our "normal" life. The shear presence and detection of microbials in our immediate environment does not necessarily mean harm or that this will cause suffering or illnesses. Obviously most of us in the medical field think that healthy individuals should be able to "handle" a certain amount of toxic or allergenic molds for brief periods without suffering any long-term harm and consequences, but we do not know for sure what are "safe" exposure limits (such as used in the chemical field with so-called "permissible exposure limits (PELs)") for various fungi or its by-products. Furthermore, medical tests are not readily available to diagnose mold-borne diseases and we are not able to provide "magic remedies" other than advice about exposure cessation and give supportive measures (which incidentally has worked for most people - see Johanning and Landsbergis, 1999 in this book). We need to improve our understanding of atypical, unusual or abnormal conditions under which naturally occurring biological materials, such as fungi and bacteria, can turn out to be a significant health risk to humans or animals. And further, when does it deserve our attention or require medical and public health intervention? All this will require interdisciplinary professional team work, consensus development, and financial support - as well as an open and honest scientific debate. We hope that this conference and this book will serve as a basis for this - so that in the end our patients and public health will benefit from this cooperative effort.

Again, I wish to thank all of our supporters, the organizing committee and the organizations that gave us their endorsement:

Mount Sinai School of Medicine, Division of Occupational and Environmental Medicine.
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International Commission on Occupational Health - Committee on Organic Dusts,
Finnish Institute of Occupational Health,
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Association of Occupational and Environmental Clinics,
National Association of County and City Health Officials,
American Lung Association.

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SPECIAL GREETINGS:

JOSEPH FORGACS, Ph.D., NEW YORK, U.S.A.

I wish to thank Dr. Eckardt Johanning and his staff for inviting me to attend the Third International Conference on Bioaerosols, Fungi and Mycotoxins. I have been interested in mycotoxicoses since 1951 and have lectured on this topic at conferences, universities, government agencies, commercial establishments, and even internationally, but, I have never seen as many individuals from a variety of countries attend a meeting on this subject as at Saratoga Springs, New York. I attended every session and read all of the abstracts with great interest, examined the poster exhibits, and I am highly impressed with the quality and depth of the presentations.

In the past, most of the mycotoxicoses described in the literature have been associated with ingestion of toxic fungal substrata, primarily by animals. The material presented here clearly indicates the importance of toxic fungal aerosols in the health of humans. Fungi include not only *Stachybotrys chartarum (atra)* but other fungi as well.

Since mycotoxins are present in spores and other morphologic structures, some of, which may be carcinogenic, inhalation of such mycologic aerosols is of paramount public health significance and certainly deserves intensive study. The relatively recent report of pulmonary hemorrhages in infants in Cleveland, Ohio suggests that *S. chartarum* in the environment may be a causal agent. Reports of this study have created an intensive interest in pneumomycotoxic research.

Levitskii and Koniukhova (Veterinaria 24: (10) 40-43) in 1943 described methods for isolation of *S. atra (chartarum)* from tissues taken from animals that died from this toxicosis. Presumably, at least some strains of *Stachybotrys* are pathogenic. Therefore, necessary steps should be taken to avoid inhalation of spores of this and other fungi while working in the laboratory and under field conditions.

In one of the reports, it was indicated that when pure fungal toxins (presumably *S. chartarum*) were administered to mice via the respiratory route, only transient or no toxic effects were noted. On the other hand, when toxin-containing spores of *S. atra* were administered by the same route severe hemorrhages and inflammation were seen. Certainly, this observation deserves elucidation.

That microbes may produce toxic volatile organic compounds of significant public health consequences appears to be at variance among workers in this presentation. In 1966, I examined microbiologically a sample of high moisture corn from a sealed bin on a dairy farm in Ohio. The corn, which had a lacquer-like odor, was fed to dairy cows. Milk from this herd was consumed by a youngster and subsequently developed a bizarre syndrome which leading medical experts were unable to diagnose. In desperation, the mother of the child discontinued the use of the suspect milk and replaced it with milk from another source. The child improved and subsequently regained health. I isolated an Actinomycete which when cultured on sterile, moist corn, gave rise to a strong lacquer-like odor. I was not authorized to pursue the problem further.

Of great interest is a report which states, *S. atra* produces a variety of toxins that range from the very potent trichothecene cytotoxins to a newly discovered class of novel natural products with unusual features, the "astronones." Importance of studies along this line is self-evident.

For sanitizing work areas where fungi have been studied, it has been indicated in one paper that a mixture of alcohol and a quaternary ammonium compound can be used successfully. While alcohol may be effective, 'quats' are of questionable value as disinfectants. They are inactivated by substances having the opposite charge, by traces of soaps, by metals such as iron or magnesium, and by organic matter. In the past, they have been evaluated using the AOAC Use-dilution technique, which is a laboratory test. When using the AOAC Use-dilution method, 'quats' perform well, but not when tested for on-site performance.

Another author recommends 10% bleach for disinfecting and sanitizing. While bleach is effective in sanitizing smooth surfaces, it is not effective on such porous surfaces as wood, since it will not penetrate the subsurface layers where mycelis and other fungal morphologic structures may be present. We found that organic tin compounds incorporated into paint and applied to wood surface are very effective in suppressing fungal growth.

Always test a cleaning agent for on-site performance before accepting it for general use. The claims set forth on the label often times are misleading.

In conclusion, data presented in the abstracts clearly supports that mycotoxic spores or aerosols, when inhaled, can produce a pneumomycotoxicosis.

Following the successful solution of equine stachybotryotoxicosis, the Russians established a central laboratory, with supporting laboratories, for the study of mycotoxic fungi. Although it may not be feasible at this time to establish such a laboratory in the Western world, it has been amply demonstrated here that under the efforts of a capable organizer, such as Dr. Eckardt Johanning, an outstanding meeting was organized with international specialists participating in assorted fields left us with valuable knowledge of various bioaerosols, fungi and mycotoxins.

PROF. EMERITUS H. BRUNO SCHIEFER,

UNIVERSITY OF SASKATCHEWAN, CANADA.

The oldest report of *stachybotrys*-induced deaths is in the Book of Exodus and took place around 1260 B.C. In the description of the "Plagues of Egypt," the 10th plague is of particular interest. It is described that the first-born children under one year of age all suffered and died. This is due to the fact that the first-born received double the ration of *stachybotrys*-infested baby food (TV broadcast, April 1998).

The second report found (Danko, 1975) indicates that in the 13th century A.C. the mongol invasion of Russia was halted by the numerous deaths of Tartar horses. According to the descriptions, they appear to have been struck by stachybotrytoxicosis.

The tragic events in Russia, Ukraine, Hungary, Romania, Bulgaria and Czechoslovakia in the 1940s-1970s are also well documented as far as the cause and deadly outcome due to *S. Charatarum* (*S. Atra*) is concerned.

However, what about the American continent? When I moved to Canada in 1969, it appeared that stachybotrytoxicosis had vanished. I knew that Forgacs had published about the disease, but the cases mentioned were all European cases, except the experimental cases by Forgacs. It is astonishing to read the extensive literature review by Forgacs (1972) which describes every detail of the east-European disease and gives all the references, as far back as 1930.

In 1984 (Auger, 1995) worked on the first massive occurrence of the disease which appeared in Quebec. Then, a few other reports appeared, mentioned in my last description (Schiefer, 1995).

While the first meeting of the International Conference in 1994 (published in 1995) was another hesitating and cautiously-phrased one, the third conference (1998) left no doubt:

Stachybotrytoxicosis is a clearly established disease in North America. Much of the "burden" falls on the shoulders of Eckardt Johanning and other investigators, and I wish the International Conference further success.

H. Bruno Schiefer

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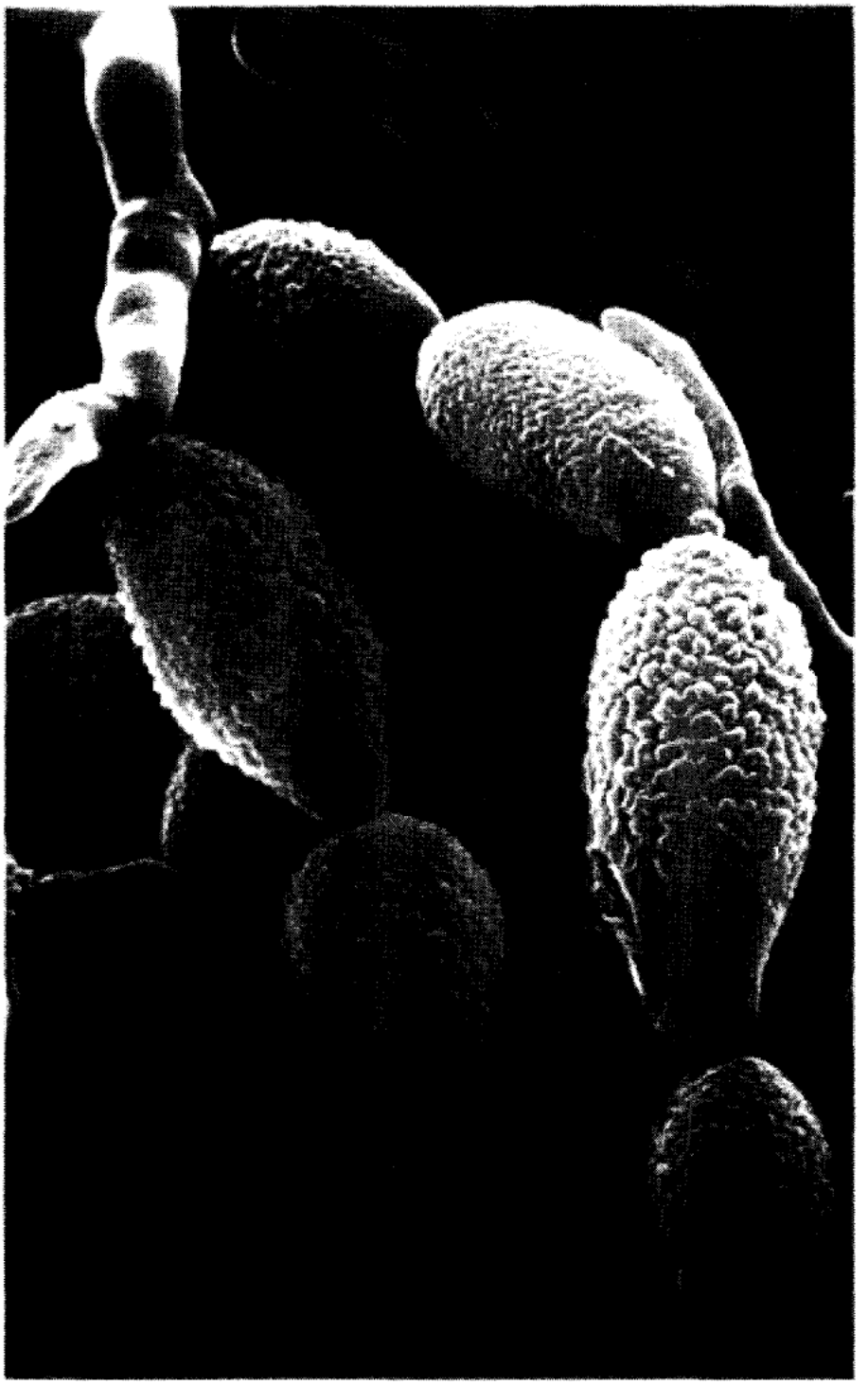

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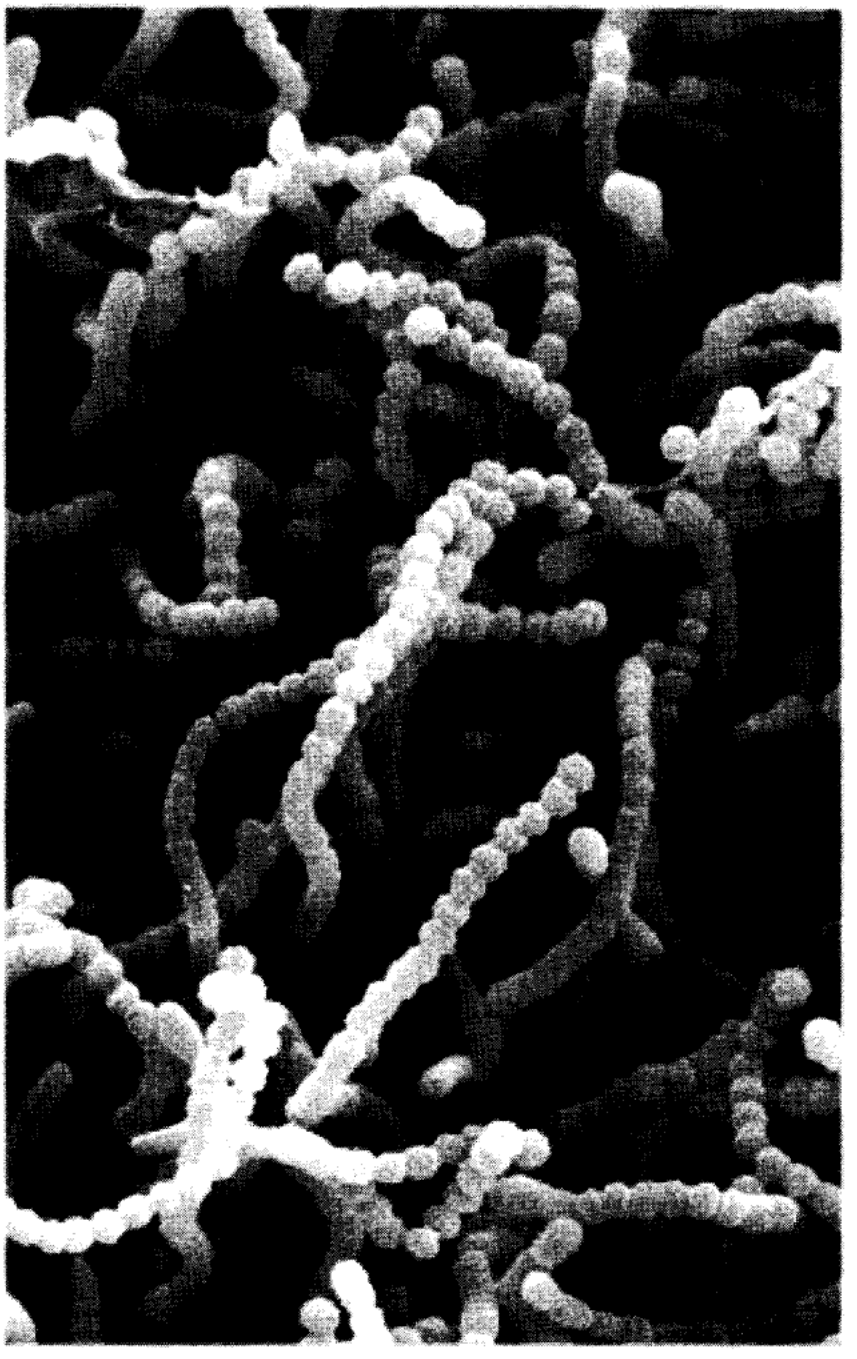

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Chapter 1

Health Effects
Pathology
Epidemiology

EFFECTS AFTER MOLD EXPOSURE - WHICH ARE THE CAUSATIVE AGENTS?

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ABSTRACT

This presentation reviews the most well known specific agents in molds and their relation to the different effects caused by exposure to molds. It is emphasized that field studies in principle only describe relationships and that such relationships not always support conclusions regarding causality for individual agents. At present, the general strategy to prevent the mold-related diseases airways inflammation, increased risk for infections, hypersensitivity pneumonitis and neurological disorders must therefore be to remove the mold contamination, viable as well as dead organisms.

KEY WORDS:

(1→3)-β-D-glucan, mycotoxins, polysaccharide

INTRODUCTION

There is general agreement that the determination of viable organisms is not a good estimate for mold exposure or the related risk. This is understandable as most of the effects are not of an infectious nature which require living organisms - but of a toxic/inflammagenic nature where the dose is determined by the biomass.

The purpose of this paper is to review some major specific agents in molds and relate them to the diseases that have been described in connection with mold exposure. The review will not deal with allergic (IgE mediated) effects as they are covered elsewhere in the symposium. Also infections in immunocompromised persons will not be dealt with.

GENERAL CONSIDERATIONS

Molds contain a number of specific agents, which can be measured in field studies. Relationships between a specific agent and effects found in such studies are never a proof of causality. Many agents - known and unknown - co-exist in molds and molds also co-exist with other agents such as bacteria and house dust mite. In an evaluation of causative agents, information from field studies is thus never complete and information on exposure to the pure agent is needed. This information can be obtained from animal or human experiments. Obviously, the human studies are more relevant but ethical considerations impose important limitations, both for chronic exposure or exposure to certain agents such as mycotoxins. We are thus facing the scientific dilemma, that although there are relations between exposure to a specific agent and effects, it is often difficult to obtain the scientific proof required for conclusions on causality.

STRUCTURAL AGENTS

Ergosterol

Ergosterol is a sterol present in the fungal cell wall and has been used to estimate fungal biomass. Relationships have been found with the extent of symptoms or spirometry, indicating a role for fungi in these symptoms [Miller and Young 1997]. There are no data which show that ergosterol might be a causative agent. It can, however, be used as a surrogate for exposure to fungal biomass or other specific agents therein.

Extracellular polysaccharides

Extracellular polysaccharides (EPS) are heat stable, water-soluble, non-branched glycoproteins and are part of the mycelial cell wall. EPS are released into the environment during growth. At present there is no evidence for a pathogenic role for EPS in fungal related disease.

(1→3)-β-D-glucan

Fungi contain a polyglucose compound - (1→3)-β-D-glucan - in the cell wall. There is an abundant literature on the biological effects of (1→3)-β-D-glucans. They can enhance the function of macrophages, neutrophils and other inflammatory cells. Systemic administration of (1→3)-β-D-glucan causes hypertrophy and hyperplasia of macrophage rich organs such as the liver, lung and spleen as well as granuloma formation [Di Luzio 1985; Williams 1997].

In animal inhalation experiments, an acute inhalation of (1→3)-β-D-glucan causes no invasion of neutrophils in the airways as is the case after exposure to endotoxin [Rylander 1997]. If an exposure to (1→3)-β-D-glucan preceded the exposure to endotoxin, a dose dependent depression of the endotoxin induced neutrophil response was found. After a five weeks exposure in guinea pigs, there was an increase in the number of lymphocytes in the airways, if endotoxin was administered together with (1→3)-β-D-glucan, but not after exposure to (1→3)-β-D-glucan or endotoxin only [Fogelmark et al. 1998]. There was also an increase in the number of subepithelial eosinophils.

Inhalation of the antigen ovalbumin during five weeks caused a very small increase in the amount of circulating IgG antibodies [Rylander and Holt 1998]. If endotoxin was administered simultaneously, there was a massive increase in antibodies but this response was abolished by the simultaneous inhalation of (1→3)-β-D-glucan. In the same experiment, the eosinophilia induced by the OVA exposure was suppressed by the simultaneous exposure to (1→3)-β-D-glucan.

In conclusion, the data from inhalation studies using pure (1→3)-β-D-glucan suggest that effects after inhalation of (1→3)-β-D-glucan are not agent-effect related but appear in form of priming, where (1→3)-β-D-glucan alters the reaction to a following exposure, be this an inflammatory agent or an antibody. The effects are found after very low levels of exposure, in the order of picograms/kg body weight. The results support a hypothesis that glucan induces a defect in macrophage control of other inflammatory cell types.

Others

A number of other cell wall products such as muramyl peptides, arabinogalactan and mycolic acids are known to be toxic. No data are available on the concentration of these agents in different environments with molds or their effects after inhalation exposures.

METABOLIC AGENTS

Enzymes

All fungi and some bacteria produce extracellular enzymes and some fungi retain these in their cell wall. Enzymes from bacteria are well known inducers of hyperreactivity and exposure to *Bacillus subtilis* proteases in industrial settings are related to asthma. Alpha-amylases from *Aspergillus* species and cellulases from Tricoderma are related to allergies among bakers. Enzymes from bacteria in the gut flora are believed to be the causative agent in mite induced sensitivity.

Volatile organic hydrocarbons

During growth, molds emit several volatile agents (MVOC) of which a number has been identified. The emission of MVOC requires living colonies of the organism and is no measure of biomass. Several of these chemicals are irritating and have been shown to cause airways inflammation in rodents when inhaled in high amounts. So far, no field studies on the relation between exposure to MVOC and effects have been reported. Human inhalation studies of the agents in environment related levels have not been published.

Toxins

Mold toxins are non-volatile, low molecular weight metabolites produced during fungal growth. Sources within the fungus are the mycelium, the extracellular matrix or the spores. Probably all fungi produce one or several different kinds of mycotoxins, provided that growth conditions are suitable.

Mycotoxins interfere with the cell metabolic processes and are carcinogenic, neurotoxic, and teratogenic [Samson 1992]. These effects probably require metabolism of the mycotoxin via the P450 system in the lungs, the liver or the gut. Ingestion is by far the most common exposure route where effects in humans have been described. Most of the few cases of possible airborne exposure to mycotoxins are among farm workers or persons processing moldy materials or in homes with excessive mold growth.

Mycotoxin contamination has been demonstrated in homes containing *Aspergillus fumigatus* [Miller et al. 1988]. Mycotoxin-producing fungi have been found in residential buildings. Smith et al. [1992] identified mycotoxins in 83 fungal isolates from damp buildings in Edinburgh. Evidence of cytotoxic materials was present in 47% of the samples. In a study of 52 Canadian homes, evidence of *Aspergillus fumigatus* mycotoxin production was found in three of the homes. Neurotoxic effects have been related to indoor exposure [Auger 1995].

COMMENTS

This review illustrated that molds contain a variety of agents with potent biological activities, either by themselves through direct contact with the cells, via metabolism or by altering the defense system against other agents.

It is well known that exposure to molds may cause a variety of medical effects, ranging from non-specific airways inflammation (probably the most common effect) to allergic reactions, lymphocyte related reactions such as hypersensitivity pneumonitis, a decreased resistance to infection, neurological effects and cancer. For specific effects such as cancer in the liver, the effect can be attributed to carcinogenic mycotoxins. For non-specific effects such as airways inflammation, fatigue and headache, it is not at the moment possible to pinpoint any specific agent as such effects may be caused by several different agents.

Thus, there is certainly a great need for further research with the ultimate aim to establish guidelines for agents which express the risk more precisely than measures of spore numbers. Outcomes such as changes in the immune function of children and possible carcinogenic effects by mycotoxin exposure are certainly motive enough for this. On the other hand, while it is clear that further information on the importance of the different agents defined - and perhaps others not yet defined - requires additional research efforts, it should not be taken as an excuse, not to decrease mold exposure whenever found in higher than normal amounts and particularly when related to effects.

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ECOLOGY, DETECTION AND IDENTIFICATION PROBLEMS OF MOULDS IN INDOOR ENVIRONMENTS

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INTRODUCTION

For many people working in the building industry or those who are consulting for indoor problems, identification of moulds does not seem a problem. In their opinion mould applying disinfectants or antifungal paints can easily solve problems. Furthermore, it is often recommended to modify the construction and adding ventilation with the result that the conditions for fungal growth can be reduced. However, if we like to know the cause and the degree of biodeterioration, in particular when health implications are involved, it is important to know which organisms are causing the problem. This is particularly true for species of *Stachybotrys*, *Penicillium*, *Aspergillus* and *Fusarium*.

Identification of mould isolates or specimens is problematic for the inexperienced researcher. This is partly due to the fact that the methods for identification are still based on the phenotypical characters of the moulds, which requires many skills in microscopical observations. In addition the mould flora of indoor environments consists of some problematic genera such as *Penicillium*, *Aspergillus* and *Fusarium* of which there is no general taxonomic consensus. Even experienced mycologists might reach different conclusions hence different names for an isolate.

Several surveys of moulds in indoor environments have revealed that we are dealing with a known and relatively low number of species. To detect these relevant species it is, however, essential to apply the appropriate methods. This paper is focused on the ecology and taxonomic problems of indoor moulds also related to methods of detection.

ECOLOGY

The mould flora in indoor environments from temperate and subtropical climates is now generally known. Table I lists the common species, which can found in moderate and subtropical climates. It is emphasized that this list is not complete because some species not listed here might occur in specific situations. Very common species in indoor environments in moderate climates are *Aspergillus versicolor*, *Penicillium chrysogenum*. *P. brevicompactum*, *Wallemia sebi* and *Eurotium herbariorum*. However the common occurrence of a certain taxon strongly depends on the growth conditions in the building. There are a few species such as *Aspergillus fumigatus* and *Penicillium olsonii*, which can be common indoors but find their original habitat for example in compost for ornamentals and tropical forest soil. Indoor environments in the tropics can also contain a different flora, often determined by a major component of phylloplane species. Much research is needed here to determine the relevance of moulds correlated with biodeterioration and health problems.

TABLE I: List of common indoor moulds. *Species occurring in wet environments e.g. bath rooms; **taxa from dry environments e.g. house dust.

*Absidia corymbifera, Acremonium strictum, Alternaria alternata, Aspergillus candidus, Aspergillus flavipes, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus niveus, Aspergillus nidulans, Aspergillus ochraceus, Aspergillus penicillioides**, Aspergillus sydowii, Aspergillus terreus, Aspergillus restrictus**, Aspergillus ustus, Aspergillus versicolor, Aureobasidium pullulans, Botrytis cinerea*, Chrysonilia sitophila*, Cladosporium cladosporioides, Cladosporium herbarum, Cladosporium sphaerospermum, Chaetomium globosum, Curvularia aureum, Curvularia lunata, Emericella nidulans, Epicoccum nigrum, Eurotium amstelodami**, Eurotium chevalieri**, Exophiala jeanselmei group*, Fusarium culmorum*, Fusarium moniliforme*, Fusarium solani*, Gliocladium species*, Gliomastix murorum, Mucor hiemalis*, Mucor plumbeus*, Mucor mucedo*, Mucor racemosus*, Oidiodendron tenuissimum, Paecilomyces variotii, Penicillium aurantiogriseum, Penicillium brevicompactum, Penicillium chrysogenum, Penicillium citrinum, Penicillium commune, Penicillium corylophilum, Penicillium crustosum, Penicillium expansum, Penicillium glabrum, Penicillium olsonii, Penicillium roqueforti, Penicillium rugulosum, Penicillium solitum, Penicillium spinulosum, Penicillium viridicatum, Penicillium variabile, Phialophora fastigiata*, Phoma glomerata*, Phoma macrostoma*, Rhizopus stolonifer*, Scopulariopsis brevicaulus, Scopulariopsis candida, Scopulariopsis fusca, Sistotrema brinkmanii, Stachybotrys arum, Syncephalastrum racemosum, Trichoderma harzianum, Trichoderma viride, Ulocladium chartarum, Verticillium lecanii, Wallemia sebi***

YEASTS

Rhodotorula glutinis, Rhodotorula minuta, Rhodotorula mucilaginosa, Cryptococcus albidus, Cryptococcus laurentii

As stated elsewhere the general concept of the flora of indoor moulds is that there is a great similarity with those commonly found on food (Samson et al. 1996, Pitt and Hocking, 1997). Two important similarities are the occurrence of *Penicillium* and *Aspergillus* (and in part *Fusarium*) and that the majority of the species are xerotolerant. For a list of species with water activities the reader is referred to Frisvad and Samson (1991) or Samson et al. (1996 p. 247). Growth characters of the species are strongly related to the availability of water. Moisture in buildings and/or associated health problems are therefore signalled by specific moulds which can grow at a certain range of water-activity, and these species can be considered as indicator organisms (Samson et al. 1994). Building material with a high aw (> 0.9-0.95) usually can contain *Aspergillus fumigatus*, and species of *Trichoderma, Exophiala, Stachybotrys, Phialophora, Fusarium, Ulocladium, Aureobasidium, Phoma* and yeasts. *Aspergillus versicolor* is common on materials with moderately high aw (between 0.85 and 0.9). This species can also be present at lower aw ($\leq$ 0.85) where it can develop together with xerotolerant species of *Wallemia, Eurotium* and *Penicillium*. Xerophilic species are commonly present in dry indoor environments but can also be detected from damp houses. Their optimal growth conditions and their visible appearance are evident when water activities drop at $\leq$0.85 (Samson & Hoekstra, unpub. observations).

The hazard of moulds in food is the possible production of mycotoxins. In many indoor studies much emphasis has been given to the toxicity of *Stachybotrys chartarum*. However many other species occurring in indoor environments are also capable to produce mycotoxins and examples are given in Table II.

TABLE II: Examples of secondary metabolites which can be considered as mycotoxins produced by moulds occurring in indoor environments (partly modified from Samson et al. 1996).

Alternaria alternata: tenuazonic acid, alternatiol, alternariol monomethylether, altertoxins
Alternaria tenuissima: altertoxins
Aspergillus ochraceus: penicillic acid, ochratoxin A, xanthomegnin, viomellein, vioxanthin
Aspergillus flavus: kojic acid, 3-nitropropionic acid, cyclopiazonic acid, aflatoxin B1, aspergillic acid
Aspergillus fumigatus: gliotoxin, verrucologen, fumitremorgin A & B, fumitoxins, tryptoquivalins
Aspergillus niger: naphtho-pyrones, malformins
Aspergillus (Emericella) nidulans: sterigmatocystin, nidulotoxin,
Aspergillus terreus: terrein, patulin, citrinin, citreoviridin,
Aspergillus versicolor: sterigmatocystin, nidulotoxin
Aspergillus ustus: austamide, austdiol, austins, austocystins
Eurotium amstelodami: physcion, echinulin
Eurotium chevalieri: physcion, echinulin
Eurotium herbariorum: physcion, echinulin
Chaetomium globosum: chaetoglobosins, chetomin
Cladosporium herbarum: epi- and fagi-cladosporic acid
Fusarium culmorum: culmorin, fusarin C, trichothecenes type B, zearalenone
Fusarium verticillioides: fumonisins, fusaric acid, fusarin C, moniliforme, naphthoquinone pigments
Fusarium solani: fusaric acid, naphthoquinone pigments
Paecilomyces variotii: patulin, viriditoxin
Penicillium aurantiogriseum: penicillic acid, verrucosidin, nephrotoxic glycopeptides
Penicillium brevicompactum: botryodiploidin, (mycophenolic acid)
Penicillium chrysogenum: roquefortine C, meleagrin
Penicillium citrinum: citrinin
Penicillium commune: cyclopiazonic acid, rugulovasine A & B, fumigaclavine A & B
Penicillium crustosum: roquefortine C, penitrem A, terrestric acid
Penicillium expansum: roquefortine C, patulin, citrinin, communesins, chaetoglobosin C
Penicillium glabrum: citromycetin
Penicillium roqueforti: roquefortine C, isofumigaclavine A & B, PR-toxin, mycophenolic acid
Penicillium rugulosum: rugulosin
Penicillium variabile: rugulosin
Penicillium viridicatum: xanthomegnin, viomellein, vioxanthin, viridic acid, penicillic acid
Trichoderma harzianum: chrysophanol, koninginin A, trichorzianines A + B
Trichoderma viride: alamethicins, emodin, suzukacillin, trichodermin, trichotoxin A
Stachybotrys chartarum: satratoxins, verrucarin J, roridin E and others
Wallemia sebi: walleminol A & B

IDENTIFICATION AND PROBLEMS OF NOMENCLATURE

An important problem, which is associated with the identification of moulds, is that much confusion exists about the nomenclature of species. A well-known example is *Stachybotrys chartarum* (Ehrenb. ex Link) Hughes which is still currently but incorrectly named *S. atra* Corda in the American literature.

Changing of names or not accepting taxonomic studies in which names and synonyms are proposed is confusing. Fungal nomenclature is not stable with many genera where the taxonomy is still unclear and debatable. For *Penicillium* and *Aspergillus* where now good consensus exists about the naming of the common species, a procedure has recently been developed to protect

names in current use. In order to protect *Penicillium* and *Aspergillus* names in current use from being threatened or displaced by names that are no longer in use, and in order to eliminate uncertainties regarding their application spelling, gender, typification date and place or valid publication, a list of names was published by Pitt and Samson (1993), on behalf of the International Commission on *Penicillium* and *Aspergillus* (ICPA, a commission of the International Union of Microbiological Societies)

Identification and reference cultures

An approach to improve identification of indoor moulds is to build a set of important reference cultures as the International Commission on *Penicillium* and *Aspergillus* (ICPA) has started. A culture set of important *Aspergillus* species and their descriptions (available on Internet http://www/cbs.knaw.nl/icpa) is now available from the major culture collections. Species included in the set are *Aspergillus candidus, A. clavatus, A. flavus, A. fumigatus, A. niger, A. ochraceus, A. penicillioides, A. terreus, A. versicolor* and two species with *Aspergillus* anamorphs, *Emericella nidulans* and *Eurotium amstelodami.*

The isolates were selected to represent a wide variety of morphological and physiological characteristics of the genus. Using these living cultures, individuals can observe these characteristics under their own lab conditions. This is extremely important because some of the characteristics of the species are influenced by minor variations in media and incubation conditions. Reference cultures can also be useful in teaching mycology laboratories and this approach for reliable identifications of indoor moulds may be worthwhile to investigate

Detection and media

For the detection of moulds in indoor environments several methods are currently applied. Unfortunately some methods are not based on the characteristics of the relevant species and still many mistakes are made.

As the flora of moulds occurring in indoor environments from temperate and subtropical climates is now generally known and consists mainly of species, which can also be found on food. Therefore the recommended methods for detection and isolation have been derived from the guidelines for the detection of food-borne moulds. General-purpose media for detection of moulds from indoor environments are Dichloran 18% Glycerol agar (DG18) and malt extract agar that should be incubated at 24°C for 5-7 days. The choice of a second medium e.g. water agar is strongly recommended. For detection of pathogenic species or specific species from warm environments, an incubation temperature of 37°C can be applied.

For air sampling, the various sieve, slit and centrifugal samplers should give comparable results. However, absolute counts of fungal propagules are not the sole criterion for the remediation of the building or solving the causal association between the moulds detected and the health complaints. A good approach, which is recommended for Canada (Davies et al. 1995) is to detect and characterize significant indoor mould proliferation foci ("amplifiers"), either by direct visual inspection or, if amplifiers are hidden, by air sampling directed toward recognition of typical indoor mould assemblages from particular kinds of substrata (e.g., water-saturated cellulosic material) in order to facilitate locating the colonized material. As a consequence, the current Canadian guidelines state that "the visible presence of fungi on moldy ceiling tiles,

humidifiers, air supply ducts, or other surfaces (including microscopically visible moulds in humidifiers) requires investigation and remedial action regardless of the airborne spore load.

Methods for the detection of visible presence of indoor moulds, which mostly consist of preparing a simple microscopical slide or using swabs from surfaces, should be emphasized more than the quantitative estimation of the viable airborne propagules. Since *Stachybotrys* produces wet slimy spores rather than dry, easily airborne propagules, these procedures also facilitate the direct detection in the great majority of cases with *Stachybotrys* problems.

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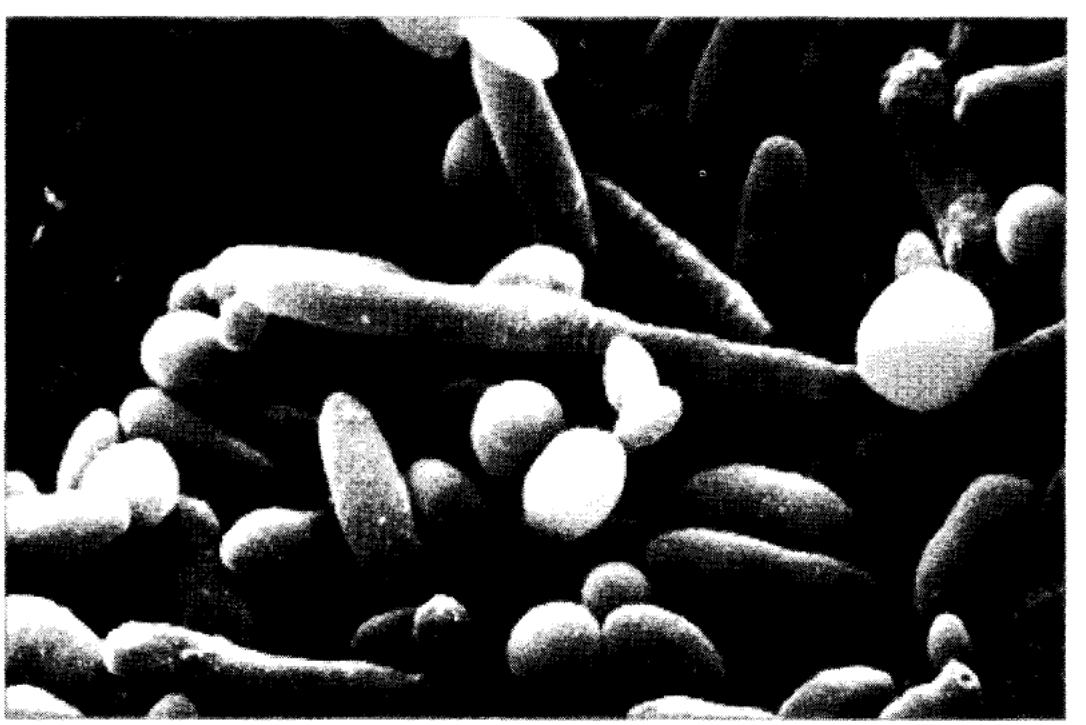

Cladosporium conidia, EM 5,400 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands.

OCCUPATIONAL EXPOSURE TO MOLDS, DISEASES AND DIAGNOSIS

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ABSTRACT

Exposure to molds has become a significant health risk to an increasing number of workers in various occupations throughout the nations. Fungal antigens are able to cause occupational asthma, rhinoconjunctivitis, hypersensitivity pneumonitis and organic dust toxic syndrome (ODTS) for instance among farmers, construction workers, housekeeping personnel as well as factory and sawmill workers who have to handle moldy and contaminated materials. In recent years, an increasing incidence of mold-induced diseases has been encountered in moldy contaminated water-damaged buildings. This has occurred both in homes and workplaces. Symptomatic persons occupying moisture problem buildings may develop asthma, rhinitis, ODTS and HP. However, the majority of the exposed individuals present with conjunctival, respiratory or systemic reactions the immune mechanisms of which remain unclear. In the present summary some aspects will be presented on health effects which appear among workers exposed especially to fungal antigens.

KEY WORDS:

allergy, occupational, respiratory, molds, moisture problems

OCCUPATIONAL RESPIRATORY DISEASES CAUSED BY FUNGI

Occupational asthma

In occupational asthma (OA) reversible airway obstruction and/or increased bronchial responsiveness follow after an exposure to specific agents (e.g. fungal antigens) which can be encountered in the workplace (Newman Taylor 1980, Chang-Yeung and Lam 1989). There are only few reports of epidemiological data on occupational exposure to microbes provoking bronchial asthma. As an example of the incidence of occupational asthma due to mold exposure in Finland in 1992-96 is shown in Table I.

The data on new cases of occupational asthma due to molds (Table I) has been collected from the official register of occupational diseases in Finland established by the Finnish Institute of Occupational Health (FIOH). The data of newly detected cases of occupational diseases has been collected 1) from all the claims which have been sent to insurance companies, 2) from newly reported cases of occupational diseases collected by the Finnish Occupational Safety

TABLE I: Occupational Respiratory Diseases Caused by Moldy Dust in 1992-96 in Finland.

Disease	1992	1993	1994	1995	1996
Allergic rhinitis	4	8	15	45	99
Asthma	3	5	13	48	84
Hypersensitivity pneumonitis					
- farming	112	137	131	87	78
- others	12	12	3	22	16
ODTS					10
Total	131	150	159	180	271

(The population of Finland = 5.15 million, people at work = 2.15 million)

Administration and 3) by adding all new cases of occupational diseases which have been diagnosed by the nationwide Institute of Occupational Health.

The annual incidence of occupational asthma caused by an exposure to moldy dusts has increased from 3 to 84 new cases in 1992-96 (Table I). Previously the majority of new cases with mold-induced occupational asthma were diagnosed among farmers handling moldy hay. During recent years, a significant increase in the incidence has been explained by the new cases of asthma detected in moisture problem buildings.

Occupational rhinitis

Exposure to fungal antigens may also lead to an allergic reaction in the upper respiratory tract causing rhinorrhea, running of nose and sneezing which often are associated with conjunctival symptoms. In the previous literature, specific allergy to fungal antigens (allergens) have been reported in many species of fungi. Mold-induced occupational rhinitis have been documented in several occupations. According to the register of occupational diseases, there has been a significant increase in the incidence of mold-induced occupational rhinitis (Table I). In 1992-96, the incidence increased from 4 to 99 new cases of mold-induced occupational rhinitis in Finland.

Hypersensitivity pneumonitis (HP) (Allergic alveolitis)

HP is an allergic lung disease which may develop in several occupations (Table II). In mold-induced HP, inhalation exposure to moldy dust leads to allergic bronchiolo-alveolitis, the diagnostic criteria of which have been presented elsewhere (Table III) (Terho 1986). Cough, dyspnea and fever appearing 4-6 hours after the inhalation of microbial antigens as well as restrictive ventilatory defect with a reduction of diffusion capacity associated with symmetric micronodular infiltration in lower areas of lungs in chest x-rays are typical findings in an acute form of mold-induced HP. Specific IgG antibodies to fungi (Table IV) can be detected in patient serum. Granulomatous inflammation and predominance of lymphocytes in lung tissue as well as lymphocytosis (mainly T helper cells) in lung lavage samples confirm the diagnosis. Usually HP does not lead to significant irreversible lung disorders if the exposure can be avoided (Kokkarinen et al. 1996).

TABLE II: Different forms of HP by etiology (Schatz and Patterson 1983, Salvaggio 1987).

Disease	Antigen source	Antigen
Bagassosis	Moldy sugargane	*T. sacchari, T. vulgaris*
Farmer's lung	Moldy hay and corn	*F. rectivirgula, T.vulgaris, Aspergillus* sp
Humidifier lung	Humidifier water	*T. candidus, T.vulgaris.*
Mushroom worker's lung	Moldy compost	*F. rectivirgula, T.vulgaris.*
Cheese washer's lung	Moldy cheese	*A. clavatus*
Cheese worker's lung	Cheese mold	*P. casei*
Dry rot HP	Moist wood	*Merulius lacrymans*
Humidifier lung	Humidifier water	*Cephalosporium* sp
Japanese summer-type HP	Home environment	*Cryptococcus* sp
Malt worker's lung	Moldy malt and barley	*Aspergillus* sp
Maple bark stripper's lung	Moldy maple bark	*Cryptostroma* sp
Sauna taker's lung	Contaminated water	*Pullularia* sp
Suberosis	Moldy cork	*P. frequentans*
Lichen picker's lung	Moldy lichen	*A. terreus, A. f*

TABLE III: Diagnostic Criteria for Farmer's Lung Disease (Terho 1986).

Main criteria:

1) exposure to microbial antigen (history, microbiology, antibodies)
2) symptoms
3) chest x-ray abnormalities

Additional criteria:

1) basal crepitation
2) impairment of DLCO
3) PaO2 decreased
4) restriction
5) histology
6) provocation

(All of the main and 2 additional criteria=>FL)

TABLE IV: Efficiency of some microbial antigens to detect precipitin positive farmer's lung sera in Finland. The experiment covered 49 sera from patients with farmer's lung disease. E.g M. faeni alone could detect 41% and M. faeni with T. vulgaris and A. umbrosus antigens could detect all patients with FL disease (Terho and Lacey 1979).

	Antigen (-s) n	Positive sera %
M. faeni only	20	41
M. faeni + *A. fumigatus*	31	63
T. vulgaris only	32	65
M. faeni + *T. vulgaris*	37	76
M. faeni + *A. umbrosus*	40	82
M. faeni + *T. v.* + *A. f*	46	94
M. faeni + *T. v.* + *A. u*	49	100

Farmer's lung (FL) is an example of HP among farmers who develop extrinsic allergic alveolitis while handling moldy hay. In Finland the incidence of FL has been 100-500 new cases of FL in 100 000 farmers. Due to active actions in occupational safety and health among farmers including education and changes in methods how to handle hay, the incidence of HP has decreased during recent years (Table I). While new cases of FL among farmers has declined in Finland, the incidence of HP has increased due to mold exposure in moisture problem buildings.

Organic dust toxic syndrome (ODTS)

Organic dust toxic syndrome (ODTS, toxic pneumonitis, inhalation fever) may occur after an inhalation exposure to toxic substances of microbes, endotoxins (von Essen et al. 1990, Rylander 1998). ODTS appears with fever, shivering and pains in muscles and joints, and only seldom produces pathological findings in lung function studies or chest x-ray examinations. Blood neutrophilia and an increase of eosinophilic cationic protein (ECP) has been detected in persons exposed to endotoxins. The diagnosis of ODTS is usually based on the history of environmental findings and the patient's symptoms. The number of new cases of ODTS in Finland is shown in Table I. Practically all cases of ODTS reported in the register in 1996 have been detected in water-damaged buildings.

MOISTURE PROBLEM BUILDINGS

Exposure to molds in damp houses

At the same time when the incidence and prevalence of mold-induced HP, OA and ODTS have decreased among Finnish farmers, the incidence of diseases caused by an inhalation to fungal antigens has increased in people occupying water-damaged buildings.

People spend more than 90 % of their time indoors in tightly sealed, poorly ventilated and overheated buildings. An increase in the incidence of allergic disorders has raised the question of a causal relationship between poor indoor air quality and allergic disorders. Exposure to allergens - including microbes - in indoor environment increases the risk to develop allergic rhinitis and asthma. What happens if indoor air is contaminated with microbes, e.g. bacteria and fungi, due to moisture problems in building materials? The exposure to microbes may increase the risk of allergic diseases, e.g. mold-induced asthma and rhinitis. However, it is possible that there are other mechanisms than allergic inflammation in symptomatic persons which can explain the pathogenesis of disorders associated with the moisture problem buildings.

Moisture problems have been encountered with an increasing frequency both in family houses and in workplaces in Finland. According to recent investigations in Scandinavia the proportion of damp houses needing an instant repair is over 50 % of all buildings (Partanen et al. 1995).

Most of the fungi and bacteria in moisture problem buildings originate from the outdoor environment. The indoor environment and building materials with increased humidity are fruitful habitats for certain outdoor microbes. The microbes do not need any special requirements to grow; practically water is all that the fungi and bacteria need. When the microbe begins to grow

it produces hyphae on the surface of the building materials. At the time of poor conditions (draught and low temperature) molds develop spores which can be sent to new frontiers in order to find other moist environments for their growth. Inhalation exposure of persons occupying water-damaged buildings occurs at the time of sporulation (emergence of spores from the hyphal fragments).

A list of typical fungi and bacteria found in moisture damaged buildings in Southern Finland is presented in Table V.

TABLE V: Microbiology of moisture problem buildings in Finland (over 350 workplaces in Southern Finland, 1996-98).

Material specimens

Aspergillus sp., *A. versicolor, A. fumigatus, Acremonium* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma viride, Stachybotrys atra, Phoma* sp., *Phialophora* sp., *Cladosporium*, actinomycetes, yeasts

Indoor air samples

Penicillium, Aspergillus sp., *A. versicolor, A.fumigatus, Cladosporium* sp, *Trichoderma viride, Sporobolomyces* sp.

People have always been exposed to fungi and bacteria in their environments. If there is an increased incidence of illnesses due to damp houses contaminated with molds, something must have happened among the exposed individuals in indoor environment. Either the quality or quantity of the microbes in indoor air has changed, or the exposed subjects have become more prone to develop diseases. At least the number of damp houses with microbial contamination seems to be higher than earlier.

Diseases in water-damaged buildings

Bronchial wheezing was more prevalent (OR 1.9-3.7) among children living in damp houses in England and Scotland than in children from houses without moisture damage (Strachan 1988, Platt et al. 1989). In Sweden, Andrae and coworkers (1988) also reported higher prevalences of chronic cough, exercise induced cough and asthma in children who had been exposed in mold houses. Bronchial wheezing (OR 1.7), cough (OR 2.1), bronchitis (OR 1.5) and asthma (OR 1.3) were more common among children living in damp houses in the USA (Brunekreef et al. 1989). In two different studies from Canada, the prevalence of asthma, bronchial wheezing, bronchitis and cough was higher in children who had reported moisture problems in their homes (Dekker et al. 1991, Dales et al. 1991).

Some of the microbes found in damp houses are able to cause allergic diseases either through an IgE-mast cell-mediated (type I, "immediate") or cell mediated (type IV, "delayed") allergy. For instance, an exposure to *Aspergillus fumigatus* in a moisture-problem building can induce sensitization and production of IgE antibodies to *A. fumigatus* antigens (allergens) (Kurup et al. 1992). After the primary sensitization, the following exposures lead immediately (in 10-30 minutes) to respiratory, dermal or conjunctival symptoms which appear as allergic rhinitis, asthma, conjunctivitis or urticaria in exposed persons. Immediate allergy due to *A. fumigatus* can be elucidated by measuring specific IgE antibodies to the microbe in the patient's serum or by using the microbial antigen in skin prick tests (Table VI and VII).

TABLE VI: Diagnosis of Mold-Induced Occupational Asthma (according to clinical experience).

Main Criteria:

1) history (micro-/aerobiology, antibodies)
2) symptoms (obstruction/wheezing.) at work
3) PEF variation at work or challenge positive

Additional Criteria

1) Ag-specific IgE antibodies
2) Skin tests +

All main and 1 additional criteria => Dg

TABLE VII: Diagnostic Criteria of Mold-Induced Occupational Rhinitis.

Main Criteria:

1) History (aero-, microbiology, abs)
2) Symptoms (allergic rhinitis) at work
3) Provocation (at work or nasal challenge)

Additional criteria

1) Ag-specific IgE antibodies
2) Skin tests +

According to the experience of the Finnish Institute of Occupational Health (FIOH), only a minority of individuals exposed to molds in moisture problem buildings develop allergic diseases. Less than 5-10 % of exposed and symptomatic workers in problem buildings present with an IgE-mediated allergy (allergic rhinitis, asthma, conjunctivitis or urticaria) and only less than 1% developed mold-induced HP. However, over 40 % of exposed individuals in these buildings complained respiratory or conjunctival symptoms associated with poor indoor air quality. More likely the microbes themselves, volatile organic compounds of the fungi, fragments of fungi or toxins cause the symptoms through a direct contact or irritation demonstrated among the exposed and symptomatic subjects.

Secondary infections

In damp houses an increase in the incidence of upper respiratory tract infections has been demonstrated especially among children (Husman et al. 1993, Jaakkola et al. 1993). Most likely the irritation of the mucous membranes of respiratory tract due to contact of fungal elements (spores and hyphae), volatile organic compounds originated from fungi and mycotoxins lead to secondary infections (e.g. sinusitis and bronchitis) due to bacteria and viruses.

Moldy materials and mycotoxins

Stachybotrys chartarum is one of the microbes found in indoor environments and especially in buildings with very damp conditions. *S. chartarum* is capable of producing highly potent toxins. In the literature, there are descriptions where *S. chartarum* has been detected in damp houses. In these buildings subjects complained headache, sore throat, flu symptoms, fatigue, dermatitis and general malaise (Johanning et al. 1996). Further investigations are needed to ascertain the relationship between *S. chartarum* and diseases in exposed patients. Contact with fragments

of fungi (i.e. spores and hyphae) and metabolites produced by the microbe (e.g. toxins) may play a significant role in the pathogenesis of diseases associated with microbial exposure in damp houses.

Even more important is the fact that *S. chartarum* is able to produce metabolites which are immunosuppressive such as cyclosporins, stachybotrylactones and stachybotrylactams. An increased incidence of respiratory infections (e.g. sinusitis and acute bronchitis) have recently been documented in damp houses (Husman et al. 1993). *S. chartarum* itself does not cause the infections but it is able to decrease the defense mechanisms of the protective barrier in exposed persons so that viruses and bacteria are able to cause respiratory disorders.

Diagnosis of mold-induced occupational diseases

The diagnosis of diseases associated with an occupational exposure to molds and bacteria in moisture problem buildings can be made by using the strategy introduced by the FIOH for the personnel working in occupational safety and health (Figure I). The investigations of moisture problem buildings contaminated with growth of molds consist of the detailed analyzing of the building itself and the microbiology of the water damaged mold materials. The clinical investigations of symptomatic persons should be carried out at the same time. Although a proportion of disorders appearing in exposed and symptomatic persons fulfill the diagnostic criteria of asthma, rhinitis or HP, these criteria should be used to confirm the most severe cases of mold-induced disorders.

Only by avoiding exposure of symptomatic individuals the symptoms can be prevented. Collaboration between different professionals, officers, occupational safety and health personnel and representatives of the workplace is important.

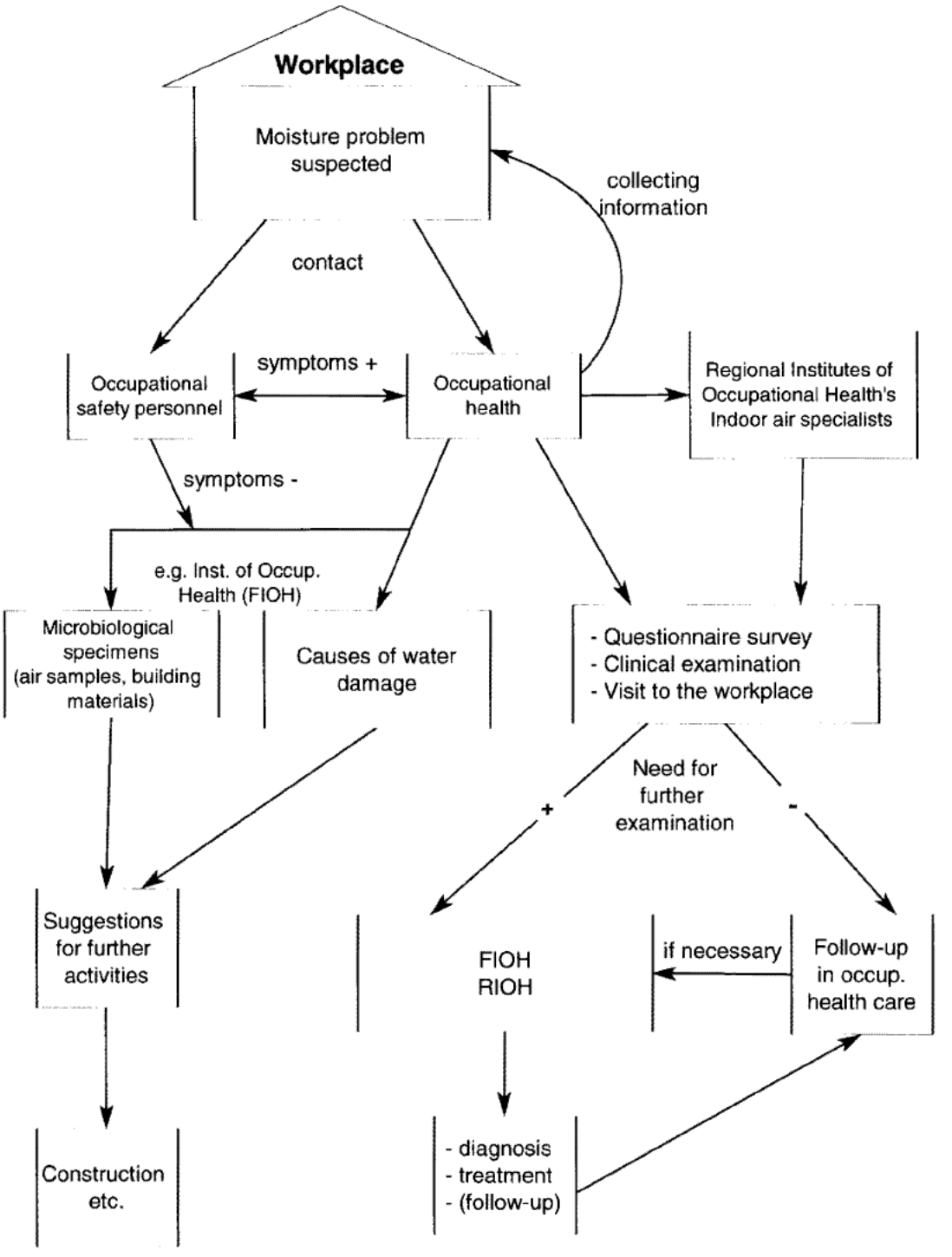
Further activities

National activities are necessary to avoid the consequences of health effects due to the exposure of molds in damp houses. National surveys both in public and private buildings should be carried out to evaluate the magnitude of moisture problems. By appropriating funds for construction activities the governments should encourage people to refurbish buildings in order to eliminate the most severe problems.

If an accidental water-damage has occurred, it has to be repaired immediately. If the signs of water-damage are visible, the primary cause of the moisture problem has to be elucidated. Only by eliminating the water-leak can the problems of molds be avoided. People often paint the spots of molds in order to hide the problem. Unfortunately, this maneuver makes the situation even worse and the problem reappears with a larger magnitude. Odors of molds are one of the first signs to warn the occupants about the risk. Microbiological examinations of indoor air samples, specimens collected from surfaces and from building materials can reveal the species of fungi existing in damp houses.

If the symptoms associated with poor indoor air quality have been reported in a damp house, health care personnel should be involved. If moisture-problem occurs in a workplace, occupational health care will evaluate the magnitude of the problem e.g. by using questionnaire surveys in problem buildings. Clinical examinations are performed as a screening method to find symptomatic individuals with diseases. Further investigations accompanied by measurements

FIGURE 1: Work strategy in relation to water-damage buildings for the personnel working in occupational safety and health.



of specific antibodies against microbes, lung function studies, provocation tests and chest x-rays should be performed in special clinics (e.g. in departments of occupational medicine) after the clinical screening.

IgG antibody levels to the most common microbes detected in mold houses are often measured from serum samples of persons who have been exposed to molds. The presence of IgG antibodies, however, only confirms a previous exposure to the microbes. Unfortunately, the role of IgG antibodies to microbial antigens still remains uncertain. At this time there is lack of commercially available test reagents or antigens which could be used in determining IgE antibodies to microbes detected in mold houses.

The fact that most of the symptomatic individuals exposed in mold houses present with non-specific conjunctival and mucosal irritation emphasizes the need for a careful differential diagnosis. The persons exposed to fungal material in damp houses and experiencing lower respiratory tract symptoms should be examined in chest hospitals or at the FIOH because several workers with allergic alveolitis and asthma have already been found at the FIOH among persons working in damp houses.

In private buildings evaluation of damp house problem should be performed by municipal inspectors or private consultants who use valid methods to estimate the severity of the moisture problem. Microbiological investigations should only be performed by professional laboratories which have the needed expertise. Primary clinical examinations should be performed by municipal health care centers in which at least one physician in each is familiar with the health problems of damp houses. After the screening, the patients should be sent to special clinics if necessary. It is important to remember, however, that the inspection of the building is the most important part of the investigation. Without knowing the extent of the problem and severity of the exposure, risk assessment can not be performed properly. On the other hand, construction activities should be launched in moisture problem buildings before the exposure to microbes develops into diseases.

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INDOOR MOULDS: A PUBLIC HEALTH PROBLEM IN BELGIUM: OVERVIEW OF 15 YEARS' EXPERIENCE

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INTRODUCTION

This paper is a summary of highlights recorded from 15 years' experience of surveys in home environments of patients with respiratory disorders linked with allergy, mainly asthma.

Actually, after the 1st oil crisis, in the 1970's, people began to renovate their house with the aim of best insulating every part from cellar to attic and we registered a continuing increase in complaints correlated with the presence of fungi, not only in damp houses but also in renovated and even new houses. Each survey responded to a specific case with different situation standards (house/flat; city/country, underprivileged/luxurious; pets/no pets....) Moreover, taking into account the evolution of the sampling methods, a standard methodology was rather difficult to elaborate.

Under these circumstances, in 1982, a scheme was set up in our laboratory at the Institute of Public Health, which belongs to the Belgian Ministry of Health, for « environmental control» in homes. It includes:

- a visit to the home,
- a standardized home environment form,
- the sampling of air, surfaces, pieces of furniture, wall paper, mattress and carpet dust for fungal moulds
- the isolation, purification and placing in our collection of fungal strains for immunological testing (more than 2000 strains are stored either freeze dried or under liquid nitrogen in the IHEM collection),
- the creation of a serum bank containing not only the serum of the allergic patient but also the sera of people living in the same surroundings,
- the standardization of a mini-method for preparing fungal extracts from each selected strain,
- finally, the immunological analysis from allergic patients, from subjects exposed to the same environment but not symptomatic and from a pool of test sera. Prick tests and antibody research (IgE and IgG) are carried out, or are in the process of being carried out, with our own extracts and have confirmed the role, in greater and greater numbers, of moulds as pneumoallergens in the home.

Our objective is to present in this article a synthesis of our work at the Institute and to inform about the interest of environmental surveys.

MATERIAL AND METHODS

At the beginning approximately 25 medical doctors collaborating with our laboratory were selected, when they were faced with patients with allergy complaints that could be linked to their home environment. The number had been increasing since then (+/-100). Actually we were called in when no answer was found with classical allergens (mites, pets...) or when the patient's home was contaminated with moulds. We have always been given the patient's medical file, and the surveys have been conducted by trained laboratory workers.

The sampling of air for culturable fungi in dwellings, offices and hospitals has been carried out using a large range of volumetric equipment. After testing different air samplers, we have selected as reference method the battery powered Reuter centrifugal air sampler (RCS+) with a HS modified medium. Surfaces are analyzed by the contact plate method or by swab. We use routinely 4 selective media, MEA for mesophilic fungi, DG18 and M40Y (Malt extract 20 gr, Sucrose 400 gr, Yeast extract 5gr, Agar 20 gr, H2O 1l.) for xerophilic fungi and TSA for actinomycetes. More specific media are chosen for particular studies like the isolation of dermatophytes in dwellings (Sabouraud + 2% Actidione).

RESULTS AND COMMENTS.

SURVEYS LINKED TO RESPIRATORY PATHOLOGIES

All our surveys, in this case, are based on thorough medical files at doctors' request. Patients are generally suffering from allergic rhinitis, asthma or even alveolitis.

We have investigated during the last 15 years more than 400 individual dwellings and flats. We will illustrate the most significant results.

ISOLATED TAXA.

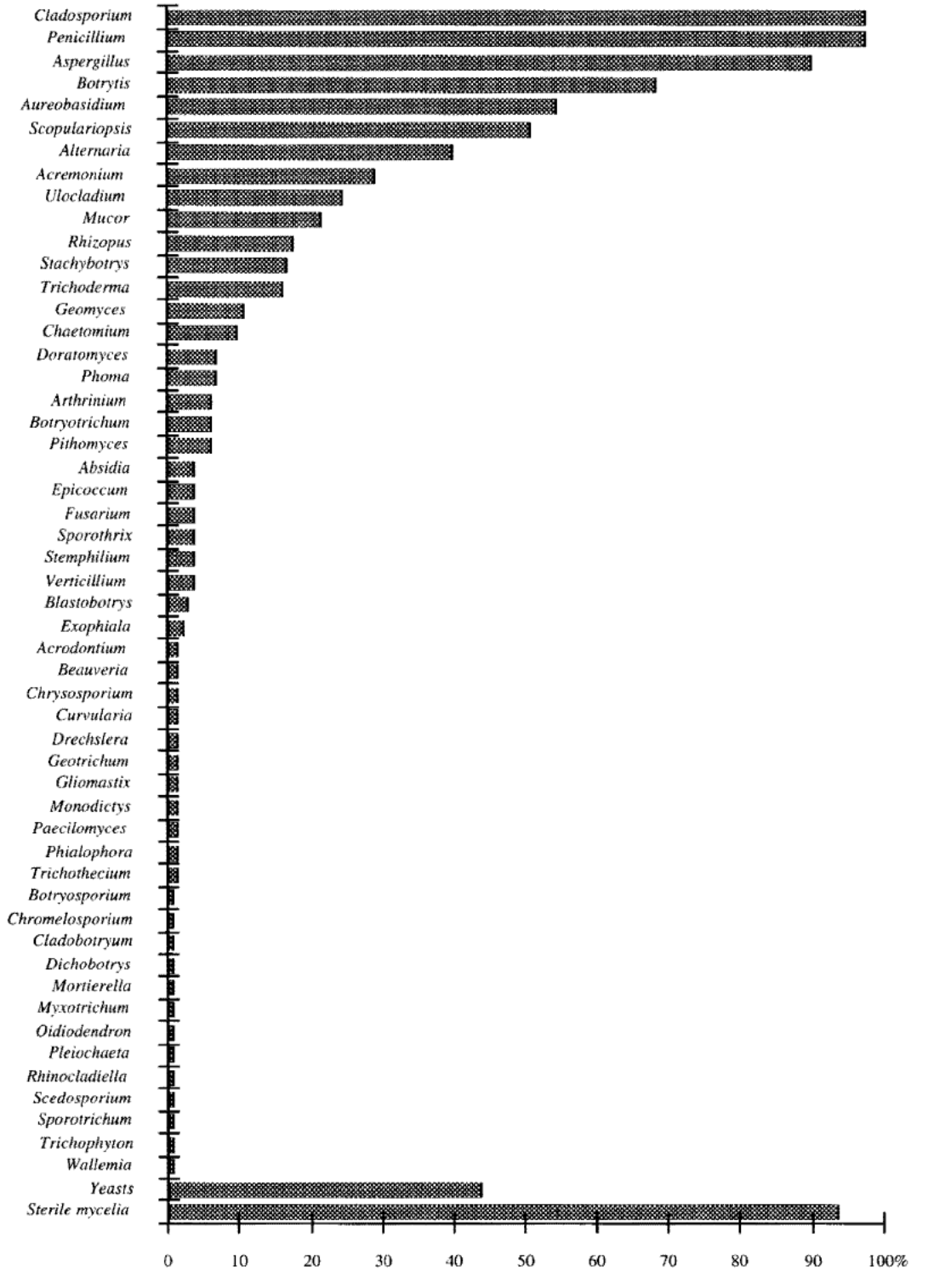
Table I shows the frequencies of filamentous fungi genera occurring in about 130 dwellings. As can be seen more than 85% of the dwellings were contaminated by *Penicillium*, *Cladosporium* and *Aspergillus*.

Cladoporium sphaerospermum and *C. cladosporoides* are the dominant species while *C. herbarum* is an outdoor mold which only invades the houses in quantities during summer entering through doors and windows. *C. cladosporoides* was mostly isolated in the basement and *C. sphaerospermum* in bedrooms, kitchens and on window frames where it is often associated with *Aureobasidium pullulans* and *Fusarium spp.*

Aspergillus fumigatus represents 45% of the total *Aspergillus* in the outdoor air but indoors, *Aspergillus glaucus* and *repens* groups are dominant. The importance of *A. fumigatus* as an indoor allergen could only be proven in houses with many contaminated potted plants.

A second group of genera includes species significantly present in about a quarter to a half of the dwellings. *Ulocladium, Alternaria, Acremonium, Aureobasidium, Scopulariopsis, Botrytis, Chaetomium, Trichoderma,* and, *Stachybotrys* could be demonstrated as playing a certain role in allergic complaints as illustrated in the following examples.

FIGURE 1: Frequencies of genera in the air of 130 dwellings



Illustrations with four examples of contamination

*Condensation is a very common problem in kitchens and bathrooms insufficiently ventilated.

Previous studies in our laboratory have shown that in kitchens, *C. sphaeropermum* is often found in old houses behind furniture. But *C. sphaeropermum* is also the first invader of new built buildings, it develops together with *Acremonium* and sometimes *Alternaria, Stemphylium* on gypsum that hasn't been painted or covered by wallpaper, on cold bridges on the external wall or even on the ceiling. In all those cases patients who did not suffer in their former house developed rhinitis or even asthma in the new one.

*In a three year old house, a child had been suffering from severe asthma for one year and could not sleep in his bedroom invaded by *C. sphaeropermum*. He was obliged to sleep on an extra bed in the living-room. The architect had asked not to cover the gypsum before it was well dried, but because of lack of ventilation the hygroscopic gypsum could not dry.

*A woman had been admitted in emergency for the third time in hospital for very severe asthma. She lived in a very luxurious house, no dampness, no visible fungi. During our survey we discovered, in the wine-cellar that, due to insufficient ventilation in this cellar, all the old bottles were covered by *Chaetomium*. Skin tests with the extract from the strain were positive. It was decided to wash out the fungi from the bottles and to place an exhaust ventilation. The woman has been healthy since then.

*Mattresses are often badly looked after.They are not vacuum cleaned, and certainly not beaten, as our grandmothers used to do. Fungal species from 130 samples of mattresses have been identified. The number of moulds varies from one mattress to another with extreme values ranging from less than 1000 to more than 70 000 000 CFU/g of dust. *Eurotium repens* is undoubtedly the most frequent species *Aureobasidium pullulans, Alternaria alternata, Penicillium chrysogenum, Aspergillus penicilloïdes* and *Aspergillus restrictus* are the other main occurring species.

It is interesting to note that carpets although dirtier than mattresses do not contain more moulds, quite the contrary. (Beguin H. (1995))

Hypersensitivity or Alveolitis (Extrinsic Allergic Alveolitis) linked to house molds

*The kitchen at the back of a rather beautiful jeweller's shop was very seldom used and a leak in the wall allowed slow but massive development of *Chaetomium globosum* on the wallpaper. The patient whose workshop was just next to this small kitchen developed extrinsic allergic Alveolitis. We could stop the development of the disease thanks to some drastic measures.

*A young girl was suffering from hypersensitivity. Hidden fungal development was discovered by opening a false ceiling. The insulating material was contaminated by numerous species as well as the inner side of the floor of the patient's bedroom. Immunoblot with extract from the strains isolated in the insulation material showed a strong reaction to several species. Let us notice that the only negative reaction was against the *A. fumigatus* extract. In fact, this species was not isolated at all in the house but we tested it as it is the only species regularly tested by physicians in our country. Asymptomatic people living in the same environment show weaker reaction to certain species. The pool of donors is the reference.

A 15 year old girl from an underprivileged family was living in a very damp and unhealthy dwelling. The house was located in a very small street where the sun never shone. The most representative species isolated in this environment were: *Phialophora fastigata, Ulocladium botrytis, Trichoderma viride, Cladosporium sphaerospermum, Graphium sp* and *Geomyces pannorum*.

Immunoblot tests revealed precipitating antibodies against the majority of the specific molds from her indoor environment. Her mother and her sister, who were asymptomatic, had few precipitating lines to some of those tested fungi. Sera from non exposed persons were negative.

The diagnosis of patients affected by fungal alveolitis should thus always be considered in relation with specific fungal survey of the house. The intensity of immunological response should be compared to that of exposed but asymptomatic inhabitants.

Exhaustive surveys upon demand from companies or local authorities

This is the case of a survey in low rent blocks. In a small country town located near Brussels, in the 1970's low rent blocks of flats were built in the same precinct. Our survey started with a classical demand from a general practitioner in relation with several cases of allergy in a family. The results showed heavy mould contamination in the flat, and it appeared that many blocks were contaminated in the same way. As a consequence, the regional authorities asked then for an exhaustive study.

There are 2 types of flats. We analyzed 164 flats occupied by retired people, single persons or families with 1 or 2 children, and found only 10 flats with significant fungal contamination, only one with *Stachybotrys chartarum* on the ceiling.

The second type consists of 2 to 3 bedroom-flats generally rent by large families with low income or being often jobless.

Some flats, more often from the second type, were in a terrible state of neglect. Moreover a simultaneous survey made by building experts revealed important building defects (cold bridges, poorly insulated metal walls, flat roofs...). All the flats situated at the extremities of the buildings and under the flat roofs had fungal problems. There was very significant condensation on single glazed window, no window-sill, water runs down and the wall paper comes off. Linen has to be washed and dried in the flats and as a consequence fungi developed on the ceiling in many bathrooms. This illustrates additional effects of building defects, social problems and high cost of electricity (thus insufficient heating)

All these parameters resulted in heavy contamination of some blocks.

The managing agency is faced with a dilemma; the best solution would of course be the destruction of the contaminated buildings, but where will the inhabitants move?

The cost of renovation is estimated at $16 million, another problem. Furthermore where to lodge the families during renovation?

And what is our role, do we have to alert population?

I think we should certainly try to draw the attention of our authorities, which has been done; indeed the problem of toxigenic fungi and particularly *Stachybotrys chartarum* has been pointed out only recently and is not yet taken into account or recognized by authorities as unacceptable

for health. The measures to be taken are still unclear, but awareness of health implications linked to this type of contamination is growing.

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PILOT ANALYSIS OF THE IMMUNE RESPONSE TO FUNGAL ANTIGENS IN SUBJECTS WORKING IN HUMIDITY DAMAGED HOUSES

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ABSTRACT.

Although the proportion of anti-mold IgG antibody producers was largely the same among different groups of subjects tested, the highest OD-readings in EIA technique were seldom seen in symptomless controls, but generally in association with any allergy of the "mold exposed" subjects or in patients referred to specialist analysis at the Oulu University Hospital outpatient clinic for oto-rhino-laryngology. Some subjects in each group were distinctly multi-immune ("responders" = R) and some just occasionally reactive ("low responders" = LR) against a panel of 12-16 mold antigens; of the 34 allergic subjects 6 were R and 3 LR. In vitro cultures of patient lymphocytes usually responded with a lower cytokine production to *Aspergillus fumigatus* antigen than to other mold antigens, in contrast to responses of lymphocytes from healthy controls. More often than not, R lymphocytes showed higher cytokine production than did LR lymphocytes; more frequently in healthy controls than in patients.

KEY WORDS:

Anti-mold IgG antibodies, lymphocyte stimulation by mold antigens, anti-mold immunity, anti-mold immunity and health impairment, mold-induced cytokine excretion in vitro

INTRODUCTION.

The demonstration of specific antibodies is generally considered insufficient to prove on the individual level that health impairment of people in humidity damaged houses is caused by exposure to molds. However, immunological laboratory tests may be developed to facilitate specific diagnostics, as mycotoxins and mold antigens have been shown to affect immune responsiveness, phagocytosis, cytokine production, histamin release etc. (Sakamoto et al. 1993, Johanning et al. 1996, Nikulin et al. 1997, Hirvonen et al. 1997, Larsen et al. 1996).

It is clear that lymphocytes are involved in any encounter with foreign molecules which results in antibody production or immunosuppression. However, to our knowledge, studies have not

been published to document the effect of mold antigens on lymphocyte function in cell cultures. So we looked for differences in the *in vitro* cytokine production by lymphocytes of healthy subjects and patients undergoing clinical examination for suspected mold-induced disease.

MATERIALS AND METHODS.

Voluntary blood samples were obtained from 55 patients under investigation at the University Hospital outpatient clinic for oto-rhino-laryngology, Oulu, Finland. Their histories (filled-in questionnaires), symptoms, non-immunological laboratory analyses and clinical findings will later be correlated with the anti-mold immune reactions.

Blood samples and filled-in questionnaires were collected from 89 University employees exposed to molds in working facilities (35 symptomless, 54 reporting toxic or allergic symptoms); further 14 subjects failed to fill in the questionnaires. Blood samples were also drawn from 33 employees from a facility without demonstrable microbial abnormalities in a survey by environmental health officers (unexposed controls).

All subjects were tested for IgG antibodies (in-house EIA method) with a panel of 12 microbial antigens, some commercial (ALK-Abelló Group), some locally produced: *Aspergillus fumigatus, A. versicolor, Cephalosporium curtipes, Cladosporium cladosporioides, Fusarium solani, Geotrichum candidum, Penicillium brevicompactum, Phoma glomerata, Rhodotorula rubra, Scopulariopsis brevicaulis, Stachybotrys chartarum* and *Trichoderma viride.* A part of the material was tested also with antigens from *Aspergillus umbrosus, Aureobasidium pullulans, Micropolyspora faeni* and *Thermoactinomyces vulgaris*. The results were calculated by subtracting the optical density (OD) reading in control test (antigen absent) from the OD reading in the test with antigen. For each subject the mean of the 12 OD-values was calculated. For the treatment of various results they were classified as "low responders", or LR, when the mean was $\geq$ 355, and as "responders", or R, with the mean $\geq$600.

The lymphocytes of the patients and most unexposed controls were cultured with a few of the antigen preparations and 6-day stimulation ($C3^*$ incorporation) was recorded. From 1- and 3-day culture supernatants TH-1 cytokines TNF-α and IFN-γ and TH-2 cytokines IL-6 and IL-10 were tested throughout with commercial EIA kits from Genzyme (Cambridge, MA, USA) and CLB (Amsterdam, the Netherlands). At orientation, a limited number of samples from healthy controls and seriously affected employees had been cultured with a broad panel of antigens in different dilutions, including 3-day stimulations and measurements of 6 further cytokines.

RESULTS.

IgG ANTIBODIES.

In all three groups of subjects both R and LR individuals were found. Generally the LR subjects had very low "background" OD-values to several antigens, with just a couple of significantly elevated readings. There were also sera that could not be classified in either category. 35

subjects among the 89 mold-exposed employees did not complain symptoms associated with their work environment; nine of these reported other types of allergy: two of these were R and one was LR. Of the 54 who complained symptoms 25 reported allergy: four R and two LR. - The means of OD readings for each antibody separately revealed a trend for increased antibody levels in the allergic subjects among both the 35 symptomless and the 54 unwell employees (Table I). When the groups were combined, the mean OD values were higher in the allergics for 12 antibodies and lower for 4 (*Micropolyspora, Phoma, Stachybotrys and Thermoactinomyces*).

TABLE I: Mean OD-values for IgG anti-mold antibodies in Oulu University personnel and patients from University Hospital Outpatient Clinic for Oto-Rhino-Laryngology.

	Medical faculty								ORL clinic
	Main building + Odontology clinic				Dept. of Med. Microbiol				outpatients
	No symptoms		Symptoms		R	LR	?	Total	
	allergy -	allergy +	allergy -	allergy +					
	(26)	(9)	(29)	(25)	(10)	(16)	(7)	(33)	(55)
Aspergillus fumigatus	383.9	432.8	472	505.2	792.3	329.1	390.1	482.4	432.4
Aspergillus versicolor	453.3	665.2 *	442.3	659.1*	949.2	247.9	585.6	532.1	512.7
Cephalosporium curtipes	411.4	577.6	395.1	475.4	643.6	174.8	387.1	361.9	377.9
Cladosporium cladosp.	286.7	550.7	463.3*	479.0*	798.5	279.8	335.6	448.9*	506.5*
Fusarium solani	633.7	877	574.3	912.1*	1050.7	296.8	792.7	630.4	709.3
Geotrichum candidum	485.1	630.4	502.6	540.7	694	296.5	470.1	453.8	502.4
Penicillium brevic.[1]	185.4	339.1	291.4*	286.3*	550.9	186.9	267.1	314.2*	246.5
Phoma glomerata	242.1	246.8	268.3	242.6	341.4	102.5	206.4	197	223.3
Rhodotorularubra	477.8	654	440.7	658	927.7	239.6	594.1	523.3	490.9
Scopulariopsis brevicaulis	411.3	593.9	402.3	519.8	685.3	164.4	377.1	367.4	407.1
Stachybotrys chartarum	374.8	460.8	372.5	359.7	592	263.1	548.9	423.4	248.1*
Trichoderma viride	307	431	430.2	549.7*	700.1	230.2	401.7	409	410.7

R = subject whose mean OD value calculated from the 12 anti-mold IgG determinations (600
LR = subject whose mean OD value calculated from the 12 anti-mold IgG determinations $\leq$ 355
* = Differs from personnel without symptoms or allergy at level $P<0.05$
Number of subjects in each group in parenthesis; [1] = brevicompactum

CELL CULTURE RESPONSES TO MOLD ANTIGENS.

Optimum conditions to evaluate lymphocyte proliferation were sought using different antigen concentrations and measuring C3* incorporation in 3-and 6-day cultures. Proliferation proved to be unexpectedly infrequent, especially the "memory" type 3-day response; 6-day responses were sometimes seen in antibody-negative subjects but, on the other hand, more frequently missed in antibody-positive individuals. In an analysis of 62 cultures 37 % of antigen exposures induced proliferation (higher than twofold spontaneous C3* readings), 29 % remained unchanged and 34 % indicated cell death or inactivation.

CYTOKINE SECRETION IN CELL CULTURES.

The supernatants were collected from cell cultures after one and three days of incubation. Some cytokine release was seen in each culture supernatant although one or another might not

be released either spontaneously or by antigen triggering of the cells of an individual (patients and controls alike). Tables II and III summarize the results in various clusters of subjects, giving the means of ratios (stimulated cytokine release per spontaneous release). The ratio for any single culture is omitted. If the reading in any culture exceeded the scale of quantitation the mean ratio is indicated by > or <, respectively. Ratios below 1 indicate inhibition of cytokine production, and the occasional zero shows that in the cultures of the cluster no measurable cytokine production was found. The ratios for IFN-γ represent secretion of Th-1 cytokines; those for TNF-α are not shown but were essentially in agreement with IFN-γ. Similarly the omitted ratios for the Th-2 cytokine IL-6 showed the same pattern as did IL-10.

Table II correlates antibody production with cell proliferation and cytokine release, and shows that patient cells responded less to stimulation with *A. fumigatus* than with other mold antigens (with the exception of late release of Th-2 cytokines in the antibody producers). In healthy controls *A. fumigatus* was usually the stronger stimulant.

TABLE II: Cytokine secretion as a multiple of spontaneous secretion in lymphocyte cultures triggered by fungal antigens. Patients and control subjects are in clusters characterized by specific antibody production and lymphocyte proliferation responses. The figures indicate means of ratios of quantitated cytokines in cultures (stimulated per spontaneous).

		No.	IFN-γ		IL-10	
CLUSTER		**obs.**	**1 day**	**3 days**	**1 day**	**3 days**
+/+	Patients	7	7.3	>19.8	22.7	13.9
	Controls	4	1.6	9.1	1.2	18.0
+/- AF	Patients	14	1.5	1.1	3.2	2.3
	Controls	7	0.2	0.9	10.1	1.7
+/- non AF	Patients	19	2.2	2.9	6.6	1.3
	Controls	9	0.7	0.6	0.7	1.5
-/+	Patients	3	0.9	5.3	8.8	8.9
	Controls	1	-	-	-	-
-/- AF	Patients	9	0.4	1.4	1.0	0.9
	Controls	6	0.7	1.1	2.0	>11.3
-/- non AF	Patients	18	1.5	2.8	>4.0	4.4
	Controls	7	1.0	1.2	>3.1	2.2

+/+ Subjects with specific serum IgG EIA and with specific lymphocyte proliferation response in 6-day cell culture

-/+ Subjects without specific serum IgG EIA with specific lymphocyte proliferation response in 6-day cell culture

+/- Subjects with specific serum IgG EIA without specific lymphocyte proliferation response in 6-day cell culture

-/- Subjects without specific serum IgG EIA without specific lymphocyte proliferation response in 6-day cell culture

AF = *Aspergillus fumigatus* as the antigen

non AF = other antigens

Table III is an analysis of the significance of the R and LR traits in cytokine release. Notably among the 24 healthy controls all R individuals were antibody-positive proliferation-negative. In most instances R cells produced more cytokines than did LR cells. In the patients this was the case in 9 of 17 comparisons (53 %), while in 7 (41 %) LR cells were superior. In the controls 6 of 8 (75%) comparisons were in favor of R cells. Antibody-producing proliferation-negative control cells were poor producers of IFN-γ. Another interesting cluster is the proliferation-positive patient cells which are strong producers of IL-10, the R group also producing high quantities of IFN-γ.

TABLE III: The effect of strong immune responsiveness (R) and low responsiveness (LR) on cytokine production of lymphocyte cultures triggered by fungal antigens. Patients and control subjects are in clusters characterized by specific antibody production and lymphocyte proliferation responses. The figures indicate means of ratios of quantitated cytokines in cultures (stimulated per spontaneous).

		No.	IFN-γ		IL-10	
CLUSTER		**obs.**	**1 day**	**3 days**	**1 day**	**3 days**
+ / + and - / +	Patients R	2-3	320	>>9	**13.6**	**17.2**
	LR	2-3	2.1	10.6	**15.5**	**26.3**
	Controls R0	-	-	-	-	
	LR	2-3	0.5	26.5	0.5	0.9
+ / -	Patients R	6-10	0.8	1.2	>4.4	0.9
	LR	3-6	1.5	1.3	0.8	0.8
	Controls R	3-5	**0.0**	**0.6**	11.5	1.1
	LR	4-5	**0.8**	**0.5**	0.6	1.0
- / -	Patients R	2 of 3	-	4.3	-	-
	LR	12-17	0.8	2.3	3.4	2.1
	Controls R	0	-	-	-	-
	LR	4-8	0.9	1,1	>2.7	2.0

+ / + Subjects with specific serum IgG EIA and with specific lymphocyte proliferation response in 6-day cell culture.

.- / +Subjects without specific serum IgG EIA and with specific lymphocyte proliferation response in 6-day cell culture.

+ / - Subjects with specific serum IgG EIA and without specific lymphocyte proliferation response in 6-day cell culture.

- / - Subjects without specific serum IgG EIA and without specific lymphocyte proliferation response in 6-day cell culture

DISCUSSION AND CONCLUSIONS.

A. fumigatus antigen seemed to suppress the cytokine production of patient cells as compared with antigens from other molds. In control cells the effect was opposite. A further comparison of cytokines and cell proliferation vs. inactivation in these cultures may show whether the effect is due to cytotoxicity or specific blocking of lymphocyte activity. Another challenge to explain this effect is raised by the low antibody response to the toxic *Stachybotrys chartarum* in ORL clinic outpatients, and emphasized by the reported isolation of an immunosuppressant

from the species (Sakamoto et al. 1993). Unfortunately our series was too small to permit an analysis of cells cultured with antigens from other toxigenic molds, but the result suggests that cellular methods may have a role in the analysis of the pathogenicity of fungi causing exposure in indoor environment.

In the light of our results, the assessment of IgG antibodies to molds may be more useful than has been concluded recently in diagnosing an individual's mold-induced health impairment. The results with a broad panel of mold antigens serve in showing which antigens could be successfully applied in cell cultures to evaluate stimulatory and cytokine responses. Knowing the patient's history of any allergy might be helpful in assigning her into the categories of multi-immune (R) or low responders (LR). An R subject with high capacity of lymphocytes to respond by IFN-γ and IL-10 production is a likely candidate to have suffered from polluted indoor air. Even if we still lack a specific laboratory test for the identification of mold-induced health impairment on the individual level, the combination of the above immunological tools may assist the problem-solving in some cases.

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CAN MICROBIAL VOLATILE METABOLITES CAUSE IRRITATION AT INDOOR AIR CONCENTRATIONS?

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ABSTRACT

Sensory irritation potency of selected volatile organic compounds assumed to be of microbial origin (MVOCs) was estimated theoretically by using the data of the recent chamber experiments and the results of volatile organic compounds (VOCs) measurements in water-damaged buildings. According to the estimations, a possible risk for complaints of irritation caused by exposure to the mixtures of those MVOCs in moisture problem buildings was evaluated. Exposure to mixtures of the selected non-reactive VOCs at the theoretical airborne concentrations calculated from the data of the chamber experiments would not result in sensory irritation in humans, and, thus, microbial growth in constructions should not increase the probability of irritating symptoms considerably. The data on MVOC concentrations measured in some problem buildings also supported this hypothesis. Irritation would be expected when the airborne concentrations of single assumed MVOC approach a level of hundreds of μ g/m^3 or mg/m^3.

KEY WORDS:

indoor air concentration, microbial volatiles, sensory irritation, theoretical estimation

INTRODUCTION

Microbial volatiles (MVOCs) have recently been used as a tracer of suspected moisture and microbial problems in buildings where the occupants complain of poor indoor air quality and building-related symptoms, such as irritation (Smedje et al. 1996; Morey et al. 1997). However, those compounds that are generally considered as MVOCs also have other outdoor and indoor sources, e.g. traffic, building materials, and human activities, which limits their indicator value considerably. The presence of MVOCs in indoor air has been assumed to be associated with irritating symptoms of occupants (Burge 1990) because exposure to volatile organic compounds (VOCs) or products of chemical reactions between VOCs and oxidants may provoke sensory irritation, i.e. burning sensation in the eyes, the upper airways, and in the skin (Mølhave 1990; Wolkoff et al. 1997). However, any convincing evidence on the irritation potency of MVOCs at indoor air concentrations has not been published so far.

In this paper, we estimated theoretically sensory irritation potency of several MVOCs by using the data of our recent chamber experiments (Korpi et al. 1998) and the results of some previ-

ous field studies (Smedje et al. 1996; Morey et al. 1997). With this demonstration, a possible risk for complaints of sensory irritation caused by moisture and microbial problems in buildings was evaluated.

MATERIALS AND METHODS

Data used in the demonstration

We used the data of our previous chamber experiments on VOC emissions from biocontaminated and sterile building constructions (Korpi et al. 1998) for the theoretical estimation of sensory irritation potency of the VOCs (n=27). Those VOCs were supposed to be emitted from microbial growth in three different building constructions. The weekly average VOC emissions were converted into the theoretical indoor air concentrations by calculating the emission rate for each VOC and determining the airborne concentration of a VOC in imaginary test rooms with and without microbial contamination (surface area of 0.5 m^2). Details of the calculations are described by Pasanen et al. (1998).

Besides the theoretical indoor air levels of the selected VOCs mentioned above, the sensory irritation potency and the probability for irritation complaints were also evaluated from actual indoor air concentrations of certain assumed MVOCs measured in moisture/mold problem buildings. We used our own data measured in five water-damaged buildings (two schools, two dwellings and one office building) in the wintertime with the methods described by Korpi et al. (1998). For the sake of comparison, we also evaluated the probability of irritation complaints from the data reported by Smedje et al. (1996) and Morey et al. (1997).

Theoretical estimation of sensory irritation potency for individual VOC and probability for sensory irritation effects caused by exposure to VOC mixtures at certain concentrations

According to Shaper (1993) and Nielsen et al. (1995), the sensory irritation potency of a chemical can be determined either by an animal model (the mouse bioassay, ASTM 1984) or on the basis of physicochemical descriptors for non-reactive volatile compounds, and is expressed as a so-called RD_{50} value (=a concentration expected to cause a 50% decrease in the respiratory rate in the mouse bioassay). For non-reactive upper airway-irritants, the occupational exposure limit (OEL) and recommended indoor air level (RIL) can be estimated from the following equations:

$OEL = 0.03 \times RD_{50}$, and $RIL = (1/4 \times OEL)/10$.

In the case of low exposure concentrations of several VOCs, such as the theoretical and measured indoor air concentrations of MVOCs in this demonstration, the additivity rule should be used for the evaluation of the irritation potency of the VOC mixtures as presented by Alarie et al. (1996). A maximum recommended threshold (T) was set as follows, with the expected indoor air concentration of each VOC (Ci) in the mixture and the corresponding Ti of each VOC:

$T = C1/T1 + C2/T2 + ... + Cn/Tn$

If the sum of the equation exceeds the value 1, complaints of sensory irritation will be expected.

RESULTS AND DISCUSSION

As we showed minutely in our recent paper (Pasanen et al. 1998), any of 27 VOCs selected in the chamber experiments can not be regarded as a reliable MVOC because those VOCs might actually originate from moist sterile constructions, and microbial growth may merely accelerate their emissions into the air. The study also indicated that the theoretical indoor air concentrations of the selected alcohols, terpenes and ketones in the contaminated test rooms were only about 0.3-1.3 % higher than those in the clean test rooms. Thus, microbial growth in constructions seems to have only a marginal effect on the total VOC level in indoor air. This should be particularly kept in mind when possible health effects of microbial volatile metabolites are discussed.

The RILs for the selected VOCs and the probability for irritation complaints caused by exposure to the VOC mixtures at the theoretical indoor air concentrations estimated from the data of the chamber experiments are presented in detail by Pasanen et al. (1998). The results revealed that no irritation complaints would be expected in any test rooms, and microbial growth of 0.5 m^2 in the constructions of the test room had practically no effect on the probability of irritation symptoms.

The irritation potency for mixtures of some assumed MVOCs measured in five water-damaged buildings and in the previous studies reported by Smedje et al. (1996), and Morey et al. (1997) was evaluated in Table I. In one building in the study of Morey et al. (1997), complaints of irritation would be expected. In this case, the total concentration of 1-octen-3-ol and 2-octen-1-ol was as high as 1.2 mg/m^3. These examples indicate that airborne concentrations of VOCs possibly derived from microbial growth in damp and moldy buildings are usually so low (on the level of ng to $\mu g/m^3$) that exposure exclusively to them should not cause complaints of sensory irritation in humans. However, it should be noted that in this evaluation, irritating properties (RD_{50}) of several compounds were defined on the basis of physicochemical variables that does not necessarily give correct values, especially for compounds with complex structure and high molecular weight. This error may be large for a single chemical but importance of over- and under-estimating errors is decreasing with increasing number of chemicals in a mixture (Alarie et al. 1996). Furthermore, irritation potency of reactive compounds should not be estimated theoretically, and, thus, the irritating effect of e.g. 3-methylfuran remained unclear in this evaluation.

TABLE I: Evaluation of the probability of irritating symptoms caused by the selected VOCs measured in damp and mold problem buildings.

Compound detected in a building	Measured concentration ng/m^3	RD_{50} value mg/m^3 *(estimate)*	RIL mg/m^3	Probability for irritation symptoms (sum> 1)
Our own data:	Building 1 / 2 / 3 / 4 / 5			
a-pinene	878000/ 25688/ 3000 / 26600 / 1401	6006*	4.5	0.20/5.7*10^{-3}/6.7*10^{-4}/ 5.9*10^{-3}/3.1*10^{-4}
ß-pinene	1800 / 1637 / 200 / 2600 / 88	29122*	21.8	(8.3/7.5/0.9/12/0.4)*10^{-5}
limonene	2800 / 1135 / 200 / 2100 / 226	*4519*	3.4	(8.2/ 3.3/0.6/6.2/0.7)*10^{-4}
1-octen-3-ol	- / 834 / - / - / -	182**	0.1	-/8.3*10^{-3}/-/-/-
1-octanol	- / 315 / 600 / - / 1522	251***	0.2	-/(1.6/3.0)*10^{-3}/-/7.6*10^{-3}
2-heptanone	600 / 282 / 30 / 250 / 37	4163***	3.1	(19/9.1/1.0/8.1/1.2)*10^{-5}
2-methyl-1-propanol	- / - / - / - / 158	5499***	4.1	-/-/-/-/3.9*10^{-5}
3-methyl-1-butanol	- / - / - / - / 17	9325***	7.0	-/-/-/-/2.4*10^{-6}
3-methyl-2-butanol	- / 11308 / - / - / -	*9645*	7.2	-/1.6*10^{-3}/-/-/-
1-pentanol	- / 813 / - / - /588	8362***	6.3	-/1.3*10^{-4}/-/-/9.3*10^{-5}
1-hexanol	- / 676 / - / - / -	997***	0.7	-/9.7*10^{-4}/-/-/-
geosmin	- / - / 100 / 260 / 35	*216*	0.2	-/-/(5/13/1.8)*10^{-4}
3-octanone	- / - / - / - / 26	17586**	13.2	-/-/-/-/2*10^{-6}
Sum of the VOCs	**883200/ 42688/ 4130/ 31810/ 4098**			**0.20 / 0.02 / 4.2*10^{-3}/ 8.0*10^{-3} / 8.3*10^{-3}**
Smedje et al.1996:	Mean concentration of 96 samples			
3-methylfuran	4	x	-	-
3-methyl-1-butanol	150	9325***	7.0	2.1x10^{-5}
3-methyl-2-butanol	12	*9645*	7.2	1.7x10^{-6}
2-pentanol	38	*9907*	7.4	5.1x10^{-6}
2-heptanone	92	4163***	3.1	3.0x10^{-5}
3-octanone	20	17586**	13.2	1.5x10^{-6}
3-octanol	1	1359**	1.0	1.0x10^{-6}
1-octen-3-ol	48	182**	0.1	4.8x10^{-4}
2-methyl-isoborneol	1	*811*	0.6	1.7x10^{-6}
geosmin	2	*216*	0.2	1.0x10^{-5}
Sum of the VOCs	**368**			**0.00055**
Morey et al. 1997:	Building 1 / 2/ 3/ 4 / mean of 119 indoor air sample			
1-octen-3-ol	100 / 10700 / 8000 / **904000** / 4800	182**	0.1	0.001 / 0.107 / 0.08 / **9.04** / 0.048
2-octen-1-ol	3200 / 4400 / 11300 / **266000** / 16100	*182*	0.1	0.032 / 0.044 / 0.113 / **2.66** / 0.161
3-methylfuran	600 / - / - / - / 1800	x	-	-
Sum of the VOCs	**3900 / 15100 / 19300 / 1170000 / 22700**			**0.033 / 0.151 / 0.193 / 11.7 / 0.209**

RIL = recommended indoor air level calculated from a RD_{50} value

- the compound was not found x RD_{50} cannot be estimated because of reactivity or complex structure of a compound

RD_{50} value was obtained from * Kasanen et al. 1998, ** Korpi et al. 1999, *** Schaper 1993

CONCLUSIONS

Estimation of sensory irritation potency of the selected VOCs showed that exposure to those VOCs at expected indoor air concentrations would not generally cause irritation. The probability of irritating symptoms caused by microbial contamination in buildings would increase considerably when airborne concentrations of single VOCs, assumed to be of microbial origin, are on the level of hundreds of $\mu g/m^3$ or mg/m^3. However, as sensory irritation is concerned in moisture/mold problem buildings, the emissions of both non-reactive and reactive compounds from the whole constructions (material emissions and microbial volatiles) as well as those from other indoor and outdoor sources should be taken into consideration.

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GROWTH CONDITIONS OF STREPTOMYCES ANULATUS REGULATE INDUCED INFLAMMATORY RESPONSES AND CYTO TOXICITY IN MACROPHAGES.

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ABSTRACT

RAW264.7 macrophages were exposed for 24 hrs to the spores of Streptomyces anulatus (doses of 10^5 10^6, 10^7 spores/million cells), isolated from a moldy building and grown on 23 different kind of media. Production of nitric oxide (NO) in macrophages was induced dose dependently by spores of *S. anulatus* reaching the level from 4.2 mM to 39.2 mM depending on the composition of the medium. The expression of inducible NO synthase (iNOS) was detected in the macrophages after the exposure to the spores collected from all tested growth media. The production of reactive oxygen species (ROS) was significantly increased when compared to controls by the highest dose of the spores from two media. The growth media affected the production of cytokines as well; the highest dose dependent levels of IL-6 varied from 400 to 7500 pg/ml and the levels of TNF a from 450 to 3100 pg/ml. The amount of death macrophages after the exposure varied from 11 % to 98% depending on the growth media of the microbe. Altogether, our results suggest that the growth conditions of S. anulatus, have a fundamental role in the ability of spores to induce inflammatory responses and cytotoxicity in RAW264.7 cells.

INTRODUCTION

Epidemiological data from different countries show that exposure to variety of fungi and bacteria growing in mold problem houses can cause adverse health effects. Respiratory symptoms related to inflammatory reactions i.e. irritation, infections and asthma, are more frequent than allergy. The specific causes and the cellular mechanisms of the illnesses associated with the exposure are not yet known. Moreover, it is not known why low concentrations of many of the microbes cause adverse health in moldy houses, whereas much higher concentrations of the same microbes in outdoor air do not cause the described symptoms. We hypothesized that a likely contributing factor in the etiological mechanism of the respiratory symptoms could be inflammatory response towards specific components or products of the microbes. This would include production of inflammatory mediators such as cytokines and nitric oxide (NO), reac-

tive oxygen species (ROS) and cytokines in immunologically active cells. NO, ROS and cytokines are important mediators in host defense, but if produced in excess they may be important in the pathogenesis of several inflammatory diseases including asthma (Cluzel and Lee, 1992).

In the present study, we investigated the effects of different growing environment of *S. anulatus* on the ability of this microbe to induce cytotoxicity and production of inflammatory mediators in mice macrophages.

MATERIALS AND METHODS

Growth media and conditions

For details see Suutari et al.1998

Treatment of RAW 264.7 cells

The mouse macrophage cell line RAW264.7 (RAW) (American Type Tissue Collection; Manassas, VA) and at 37oC, 5 % CO2, in RPMI Medium 1640 (Gibco Lab., Grand Island, NY) supplemented with 10 % fetal bovine serum (HyClone Lab., Logan, Utah), 1 % L-glutamine, and 1 % PNS antibiotic mixture (Gibco Lab., Grand Island, NY). The cells were then exposed to the spores of S. anulatus (doses of 10^5 10^6, 10^7 spores /million cells) for 24 h.

Analysis of NO production

NO was assayed in the culture medium by the Griess reaction as the stable NO-oxidation product nitrite, which was quantitated spectrophotometrically using an automated colorimetric procedure (Labsystems iEMS Reader MF). Inducible NO synthase (iNOS) in macrophages were measured by Western Blot Analysis (Hirvonen et al. 1997).

Analysis of reactive oxygen species

The spores were first opsonized with foetal bovine serum for 30 min at 37oC. ROS were measured by applying a chemiluminescence assay with a Bio-Orbit 1251 luminometer (Bio-Orbit, Turku, Finland) as earlier described by Ruotsalainen et al. 1995. The dose of the spores was 2 x 10^7 spores/million macrophages.

Analysis of cytokines

TNF α and IL-6 were analyzed by "sandwich-type" enzyme immunoassay by using commercial ELISA kits (Pharmingen, San Diego, CA).

Cell Viability

Viability of the cells in the culture after the treatments was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) test (Sigma, USA) based on MTT-reaction with functioning mitochondria to produce formazan. Formazan was quantitated spectrophotometrically using automated colorimetric procedure.

STATISTICS

The data were analyzed using one-way analysis of variance and Duncan's Multiple Range test, Wilcoxon Signed Rank Test and Wilconson Rank-Sun Test ($P < 0.05$).

RESULTS

Production of NO and cytokines in macrophages was induced dose dependently by spores of S. anulatus and the expression of iNOS was detected in the macrophages after the exposure to the spores grown in all tested growth conditions. However, there were significant differences in the amount of NO produced by macrophages between different growth conditions of the spores. The production of ROS was significantly increased when compared to controls only by the highest dose of the spores grown on complex media containing yeast extract. Moreover, the growth media of the microbe had significant effect on the ability of the spores to induce cell death in macrophages (Table1).

TABLE I: Effect of different growth medium of Streptomyces anulatus (1x 10^6 spores /million cells) on the ability of the spores of this microbe to induce production of inflammatory mediators and cytotoxicity in mice RAW264.7 macrophages during 24 hrs exposure.

Mediator	**Changes in induced levels of inflammatory mediators/cytotoxicity**	**Compounds in growth medium of S. anulatus affecting the levels**
Nitric oxide (NO)	4.2 - 39.2 μM	arginine ↑, NH3NO4 ↑, glucose ↑, glycerol ↑
Reactive oxygen species	100 - 280 (% of controls)	glycerol ↑, yeast extract ↑
Cytokines: TNFα	450 - 3100 pg/ml	glucose ↑, glycerol↑, Na-propionate↓
IL-6	400 - 7500 pg/ml	- " -
Percentage of dead cells	11 - 98 %	Na-propionate as carbon source without sugar, starch
	arginine NH3↓, glycerol↑,	

DISCUSSION AND CONCLUSIONS

The present results demonstrate that the growth conditions of S. anulatus, have a fundamental role in the ability of spores of this microbe to induce inflammatory responses assessed as production of NO, ROS, IL-6 and TNF α and cytotoxicity in RAW264.7 cells. The ability of S. anulatus to induce production of these mediators is of special interest since the role of NO and cytokines as important inflammatory mediators in the non specific host-defense against microbes is generally accepted. NO is also known to play an important role in regulating airway function and in the pathophysiology of inflammatory airway diseases including asthma

(Barnes, 1995). Cytokines, such as TNF α and IL-6 are able to potentiate allergic inflammation and seem to be implicated in the development of late allergic reaction (Gosset et al. 1993).

ROS are also released by stimulated phagocytes in the airways. ROS known to be released in the bactericidal process include superoxide anion, hydrogen peroxide, and hydroxyl anion, which may exert their toxicity not only to invading bacteria but also to surrounding tissue. Moreover, it has been postulated recently that oxidized NO species may play important role in biological activity of NO, because NO is capable of rapidly reacting with ROS such as molecular oxygen and superoxide forming highly toxic peroxynitrite molecule.

Thus, these results suggest that inflammatory response towards specific organic materials in the microbes is may be among the most important contributing factors in the etiological mechanism of the respiratory symptoms. Moreover, these results support the assumption that the growth and sporulation environment of the microbes in the moldy houses enables the normally innocent microbes to produce components and/or metabolites, which are able to cause the described adverse health effects based on cytotoxicity and production of inflammatory mediators in mammalian cells.

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CLINICAL FINDINGS RELATED TO INDOOR FUNGAL EXPOSURE - REVIEW OF CLINIC DATA OF A SPECIALTY CLINIC.

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ABSTRACT

OBJECTIVE: Review of symptoms, clinical abnormalities and laboratory findings of building occupants with abnormal indoor fungal exposure, primarily allergenic and toxigenic fungi (*Stachybotrys atra, Penicillium, Aspergillus*).

DESIGN: Descriptive review of 151 cases evaluated at a specialty clinic. Analyses of a standardized health symptom questionnaire and immunological laboratory data.

Subjects: Pediatric and adult specialty clinic population with verified abnormal indoor fungal exposure.

MAIN OUTCOME MEASURES: Self-reported symptom-complexes, Immunoglobulin E and G, T- lymphocyte enumeration and function.

RESULTS: Widespread contamination of water-damaged building materials primarily with toxin producing fungi were identified in the patients exposure history, who are reporting from a variety of nonspecific"sick building"-like complaints to a history of more serious morbidity of the respiratory system, skin, eye, chronic fatigue-like symptoms (CFIDS) and central nervous system dysfunctions (vertigo, memory, irritability, concentration, verbal dysfunctions). In some cases abnormalities of enumeration and functional laboratory tests (flow-cytology), mainly of the white blood cell system were identified. IgE or IgG antibodies used as exposure markers, were positive in about less than 25% of all cases. Removal from exposure typically resulted in improvement in the majority of cases.

CONCLUSION: Indoor air exposure to mycotoxin and allergen producing fungi results in a high frequency of health complaints, variant multi-organ and laboratory abnormalities requiring a detailed exposure assessment and clinical evaluation. Removal from fungal exposure and symptomatic treatment generally results in noticeable improvement of most patients. I propose to name these presentations and clinical findings-if certain criteria are met: "fungal syndrome".

KEY MESSAGES:

Many patients with fungal indoor exposure have a variety of symptoms, primarily of the skin, mucous membrane, respiratory organs, central nervous system and constitutional symptoms.

Laboratory results suggest other than type I allergy, IgE mediated health responses.

Diagnosis of fungal disease should be based on exposure history, medical history and clinical findings.

The prognosis appears good provided the exposure can be stopped.

INTRODUCTION

Indoor exposure to allergenic and toxic fungi is gaining recognition as an important, but clinically "challenging" environmental risk factor for a variety of illnesses with public health importance (Husman, 1996; Reijula, 1996). Excessive fungal growth in buildings is often the result of building design defects, leaking roofs and walls, natural disasters like flood and storm damage, plumbing failure or defective ventilation systems and the presence of organic building materials (wood, paper) necessary for microbial amplification. NIOSH reported in 1996 that of 104 HETA building investigations about 42% had some type of water damage. (Crandall, Sieber 1996)

In the past, "mold" (fungi) related health problems and diagnostic tests have not played an important role in clinical allergy and immunology for a variety of reasons. Among many medical providers it is known, that fungi have the ability to cause infections and may be "commonly encountered allergens" causing respiratory, cutaneous or conjunctival allergy (Horwitz, Bush 1997). There is a growing number of reports linking various other illnesses with indoor contamination with fungal and bacterial products. Some of these effects are related to complex allergic and toxic reactions not explained by "simple allergy". Little has been reported in the mainstream medical literature about irritant and toxic effects of fungi and its metabolites, although among veterinarians and food-safety specialists adverse health reactions to fungal toxins are well known. Because of that, often patients now have to seek help by other specialists experienced in environmental and occupational pathology or toxicology. One fungus, *Stachybotrys chartarum (a.k.a. S. atra)*, that can produce potent mycotoxins has been associated in the past with serious adverse health effects in animals and humans in agricultural environments (Hintikka 1978). In general, high exposure levels of airborne fungi have been reported among farmers, wood workers, composting waste workers, but may also be found in defective hospitals, offices, schools or residential homes (Croft et al. 1986; Mainville et al. 1988; Auger et al. 1994, Johanning et al.1998). In recent medical- epidemiological investigations associated health problems have been described among office workers handling moldy paper materials and breathing fungal contaminated air (Johanning et al. 1998; Johanning 1998), in infants (Montaña et al. 1977; Sorenson et al. 1996; Etzel et al. 1998), and in courthouse workers (Hodgson et al. 1998). These effects are thought to be related to microbial agents including toxic metabolites (mycotoxins) produced by certain fungi, such as those in the genera *Stachybotrys, Aspergillus versicolor, Penicillium, Trichoderma* and *Fusarium*. *S. chartarum* appears clinically important, because it produces biologically very potent trichothecenes and other mycotoxins, which have been shown to interfere with the protein synthesis, on the DNA level and the cellular or humoral immune system (Corrier, 1991; Sharma, 1991). The production of mycotoxins depends on environmental conditions and substrates, and is not evident by macroscopic growth characteristics (Nikulin et al. 1994; Johanning et al.1998).

METHOD:

STUDY GROUP SELECTION AND DESCRIPTION:

This is a descriptive study of self-reported health complaints and results of clinical evaluations in an ambulatory occupational and environmental health clinic of patients coming primarily from the Northeastern United States. In this study subjects (age range 1 to 81 years, n=151; mean age 39 y, median 41 y, 65% females, 19% were smokers) were included, if they had a verifiable history of indoor exposure to fungal air contaminants and if they had completed a standardized health symptom questionnaire. Unusual fungal exposure was considered present ("positive"), if industrial hygiene surveys confirmed the presence of large amounts of atypical indoor fungi and significant moisture problems (leaks, flooding, condensation, etc.). In addition, for comparison purposes results from an earlier study are presented, which included well-documented exposure and which utilized the same core questionnaire. The earlier study included a control group of non-exposed adult population with similar demographics (Johanning et al. 1996).

HEALTH SYMPTOMS SURVEY.

The health symptoms survey had been adapted with slight modification from the Mount Sinai Medical Center Fungal Health Survey Questionnaire (Johanning et al. 1996). Symptom questions were classified according to particular organ systems: "upper respiratory system" (nasal irritation, burning, itchiness, stuffiness and congestion, nasal bloody discharge, throat irritations and soreness); "lower respiratory system" (cough, shortness of breath, wheezing, chest congestion and tightness); "central nervous system" (severe headaches, concentration problems, irritability, dizziness or lightheadedness, sleeping problems, fatigue); "eyes" (burning, irritation, blurry vision); "skin" (burning, erythematous rash, hair loss); "constitutional" (low grade fever, tender/swollen lymph nodes, flu-like symptoms) and history of previously diagnosed "allergy".

LABORATORY TESTING:

Laboratory tests were performed to study red and white blood cells, multi-chemistry and immunological parameters. Lymphocyte subgroups (CD3, CD4, CD8, CD19) were measured by flow-cytometry. The medical laboratory tests were analyzed by licensed commercial labs following standard quality assurance (Q.A.) procedures (IBT-laboratory, Kansas, Ms., and Specialty Laboratory, Inc., Santa Monica, Ca.). To evaluate possible type I allergic reactivity, allergen-specific immunoglobulin E (IgE) was tested in a panel for: *Cheatomium globosum*, *Aspergillus fumigatus*, *Cladosporium*, *Penicillium notatum*, *Stachybotrys atra*, mouse, rat and pigeon serum proteins, and American Cockroach. A panel of immunoglobulin G (IgG) was measured: *Micropolyspora faeni*, *Thermoactinomyces spp.*, *Alternaria alternata*, *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Penicillium notatum*, *Phoma herbarum*, *Trichoderma viride*, *Pigeon droppings*, *Pigeon serum*, *Bovine serum*, *Cladosporium and Stachybotrys atra*. Allergen testing material was provided by Pharmacia & Upjohn Diagnostics.

EXPOSURE ASSESSMENT:

Indoor exposure to fungi was assessed relying either on reviews of environmental survey reports provided by the patient's own consultant or by our own verification generally relying on test methods described by the AIHA field guide for the determination of biological contaminants (1996. Dillon et al. 1996). The indoor exposure was generally characterized using conventional microscopic and culture methods, but in some cases also mycotoxin cytotoxicity screening tests (MTT) was performed (Gareis, 1995; Gareis et al. 1999).

RESULTS:

In the study 151 patients participated and completed a standardized questionnaire. In addition, they underwent blood testing for immunological abnormalities. Most of the cases were related to residential home defects and work related exposure occurred in 23% of the cases. Defects were related to variety of natural or man-made causes including flooding, severe weather (storms), snow and ice damage, roof leaks and building envelop defects, wall condensation, sewage backup, earthquake and water damage caused by firefighting. A large number of cases outside the Northeastern region came from California and Florida. In the exposures assessments, some or high exposure was identified to *Penicillium spp.* (78%), *Aspergillus spp.* (76%) *and Stachybotrys atra (or chartarum)* (44%). A variety of other moisture loving fungi were also detected. Mycotoxin exposure was confirmed in 16% of the cases based on the MTT screening test results. However, it should be noted that not all cases were tested for presence of mycotoxins, because of logistical reasons. The professional and educational backgrounds of subjects included: students, teachers and university professors, office workers (governmental and private), physicians and other health care providers, lawyers, clergy, hospital workers and domestic workers (housewives).

The survey of the health complaints have been listed by symptom complexes and organ system in Table I (see also Graph I). Almost half of the respondents (46%) reported symptoms affecting both the 'nose' and the 'throat; for 'lower respiratory organ' the majority of respondents (57%) in this category reported a minimum of three complaints (either shortness of breath, or cough, or cough lasting more than three months, or wheezing, or chest tightness, or exertional dyspnea) with 17% percent reporting all six types of symptoms. In respect to central nervous system complaints (headaches, nervousness, concentration problems, dizziness, and excessive fatigue) a majority (52%) reported at least three complaint types. In the category of skin and hair related complaints (general rash, rash of hands, redness, itchy/peeling skin, cracking skin or hair loss) most respondents (42%) reported at least two types of complaints. In respect to 'eye' complaints, 62% reported only either 'burning' or 'blurry vision'. Stratification of symptom complexes by elevated IgG levels (*Aspergillus fumigatus, Penicillium notatum, Stachybotrys a.*) did not result in statistically significant trends for upper or lower airway symptoms or central nervous system symptoms.

TABLE I: Symptom survey by effected organ system of clinical cases with fungal exposure (Proportion of subjects reporting at least one symptom in each symptom complex).

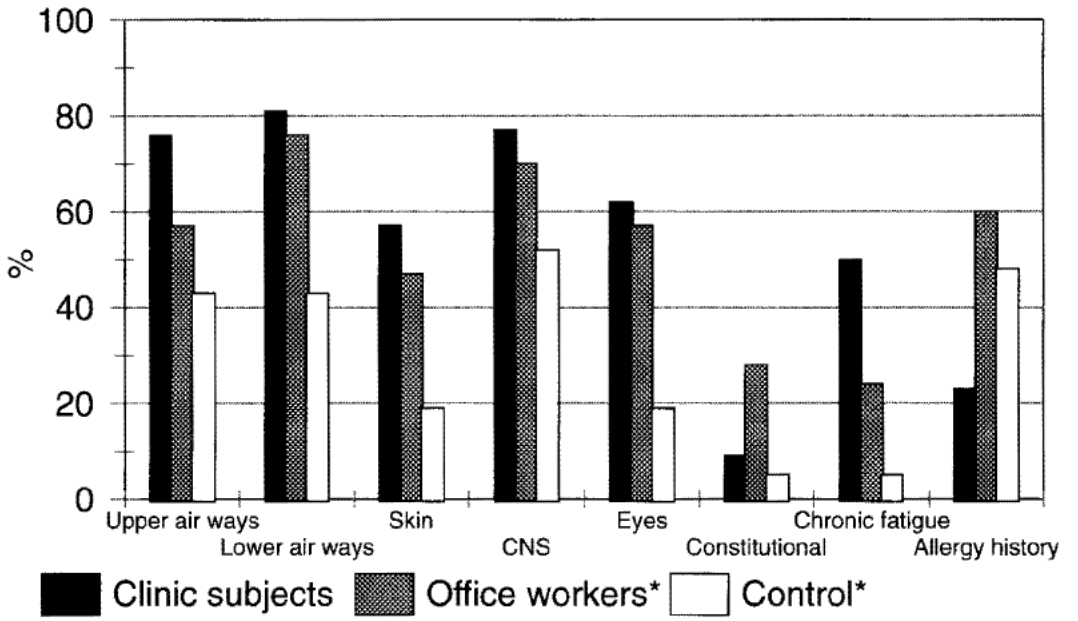
Organ system affected:	Clinic subjects n=151	Exposed Office workers* n=53	Control* n=21
Upper air ways	76	57	43
Lower air ways	81	76	43
Skin	57	47	19
CNS	77	70	52
Eyes	62	57	19
Constitutional	9	28	5
Chronic fatigue	50	24	5
Allergy history	23	60	48

* data from different study (Johanning et al. 1996)

Between 3% to 8 % had elevated IgE-antibodies to specific fungi, the highest rates were found for *Penicillium notatum* and *Stachybotrys* IgE and the lowest for Cheatomium, mouse and rat specific IgE-antibodies. Between 17% (*Stachybotrys* IgG > 39mcg) to 52 % (*Aspergillus fumigatus* IgG) of the subjects had immunoglobulin levels above the normal reference range.

Between 2-8% had T-Lymphocyte sub-population counts above the normal reference range and 0-18 % had counts below the normal laboratory reference range. Mature T-lymphocyte (CD3) absolute counts and percentages below the normal reference range was detected only in 7 or 2% of the tested cases respectively. None of the tested subjects had a CD4/CD8 ratio that was below the laboratory normal range. 18% had a natural killer cell (%) below the normal reference range. If laboratory abnormalities of tested subjects are stratified by exposure categories based on hygiene survey information, the following trends were detected. If there was evidence for *Penicillium* exposure only 3% ($p<.029$) had an *elevated* CD4% -lymphocyte levels above the reference range versus 40% that had no evidence for significant *Penicillium* exposure. A similar trend was recognized for mature lymphocytes (CD3%) being *below* the reference range with 0% (with PNC exposure) versus 20% (without PNC exposure) ($p<.08$, Fisher's exact test). A similar association of exposure with fewer abnormalities was noted for the *Aspergillus* exposure category with *elevated* CD4 % levels of 40% (for those without ASP exposure) vs. 3% (with ASP exposure) ($p<.029$, Fisher's exact test) and CD3% levels below the laboratory normals with 20% (without ASP exposure) and 0% (with ASP exposure) respectively ($p<.081$). For the *'Stachybotrys"* exposure category no significant statistical trends of laboratory abnormalities were detected. However, if the MTT cytotoxicity test was positive (exposure category 'mycotoxin test positive') the proportion of total IgG counts (33% vs. 3%, $p<0.07$, Fisher's exact test) and white blood counts (11 vs. 0%, $p< .08$, Fisher's exact test) above the reference range higher than for those not exposed. The proportion of elevated absolute mature T-lymphocyte counts (CD3#) above the reference level was also higher in the mycotoxin exposed group (22%) than those not exposed (0%) ($p<.006$, Fisher's exact test).

FIGURE I: Symptom survey by affected organ system of clinical cases (n=151) with fungal exposure compared with previous study results in which office workers had significant allergenic and toxigenic fungal exposure (see Johanning et al. 1996).



* reference study (Johanning et al, 1996)

DISCUSSION:

The purpose of this study was to present an overview of the "typical" clinical presentation of patients with health problems related to fungal indoor exposure as encountered in a clinical specialty practice. The results of these patients who sought medical treatment because of illnesses associated with documented fungal indoor exposure suggest a similar trend of adverse health reactions as has been previously reported by this and other authors (see this proceedings). Primarily inflammatory effects of the upper and lower airways, skin and mucous membranes were detected, but also systemic effects involving the central nervous system (brain function), and constitutional reactions such as extreme chronic fatigue. The laboratory results suggest that fungal and mycotoxin exposure may have an effect on the T-lymphocyte immune response, although a clear trend of clinically detectable immune suppression or modulation could not be identified in the studied sample. This may be related to the heterogeneity of the study group in respect to exposure and time of laboratory testing. Some of the associations between exposure and laboratory abnormalities may also be due to multiple comparisons. This study has methodological limitations because of its design (cross-sectional survey of data collected over time) and does not claim to prove causation for specific diseases and fungi for which more rigorous research would be required.

There are several possible reasons why the laboratory findings did not show many "strong" or "significant" trends and abnormalities. There was no control group included of people without any fungal (microbial) exposures, because of logistical and financial reasons. All patients were exposed to some degree to indoor fungi without significant variation. Therefore we may have not found strong associations between them and other variables. Exposure was not measured at the time of the laboratory testing. There were few patients with comprehensive immune function tests above or below the reference range, which may be related to the delay of testing after exposure was identified and the variability of the human immunology over time and between different subjects. With the small number of lymphocyte testing abnormalities in the relatively few tested subjects finding an association with exposure is statistically difficult. In respect to IgE and IgG and immune function tests we looked in this study only at test scores below or above the reference ranges (categorical value), but not actual values (continuous value). Continues values may provide greater statistical power to observe an association, if one really exist. We are currently preparing such an analysis for a subsequent study.

This study is made up of "mixed bag" exposure conditions and several dominant fungal species found in the environmental surveys. There may have been other microbial agents or chemicals, such as endotoxins, beta-glucans and MVOCs, for which we have no exposure data, but which may be an important variable. However, for all that all subjects it is common, that they had developed intolerable or complicated health problems, that prompted them to seek specialty medical care. Many of these subjects reported that their local health care provider felt limited or not able to assist them in the investigation and successful treatment of their conditions.

The precise exposure conditions and duration varied between the subjects because of many factors such as different climactic conditions, microbial growth characteristics, unique building structure features, variable visible or hidden fungal growth area, and the delay in getting medical attention and instituting corrective measures.

A comprehensive blood analysis of subjects and tests of fungal toxin production (MTT cytotoxicity screening) was not possible in all cases because of either lack of availability and access to appropriate testing materials. The natural fungal growth conditions and air contamination levels showed considerable variation in the investigated cases. The fungal toxin exposure of building occupants and the associated health reactions may therefore vary from case to case depending on several factors, including susceptibility, prior sensitization, climactic and building conditions.

In a subsequent preliminary follow up study (telephone interview) the majority (68%) reported, that their had health significantly improved or the illnesses resolved after the exposure to the fungi was stopped or reduced, either because they left the contamination area (74%) or because of remediation efforts and repair of building defects (21%). Subjects who reported that "nothing was done" (6%) also reported that they had no significant improvement in their health status.

Systematic studies with improved standardized exposure assessment, more specific and sensitive biological markers, comprehensive health indicators and improved epidemiological design are needed to better understand indoor fungi associated disease and immunological changes over time. Based on the present knowledge about the toxicology and allergy, the physiological

and pathological findings, the observed multi-organ health reactions associated with indoor fungal exposure, they clinical findings could be best described as a "fungal syndrome". The clinical presentation appears to depend on the fungal species, the intensity and duration of exposure, a variety of host factors, and the time of medical intervention. Clearly a IAQ related "fungal syndrome" diagnosis should only be made if adequate exposure information is available, a comprehensive medical and occupational/environmental history has been obtained, and a complete medical evaluation (complemented by supportive laboratory testing) was done. Mycotoxicosis studies will be improved when reliable and sensitive biological markers and methods are available to detect the various mycotoxins in serum or urine (Dietrich et al. 1998).

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PULMONARY HEMORRHAGE AMONG INFANTS WITH EXPOSURE TO TOXIGENIC MOLDS: AN UPDATE

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ABSTRACT

In 1994, we investigated a geographic cluster of 10 cases of acute idiopathic pulmonary hemorrhage among infants in Cleveland, Ohio. Our matched case-control study demonstrated that infants with this condition were more likely than control infants to live in homes with toxigenic *Stachybotrys chartarum* and other fungi in the indoor air. The risk appeared to increase when both *S. chartarum* and environmental tobacco smoke were present in the home. Since the completion of the 1994 investigation, 27 additional infants in the Cleveland area have been diagnosed with acute idiopathic pulmonary hemorrhage. Of the 37 infants, 30 were African-American infants, all of whom lived in a limited geographic area of eastern metropolitan Cleveland, an area of older housing stock. Twelve deaths with extensive idiopathic pulmonary hemosiderosis have been identified, including seven originally thought to be due to Sudden Infant Death Syndrome (SIDS).

To determine how common this problem might be, we actively solicited reports of idiopathic pulmonary hemorrhage in infants from pediatricians throughout the United States. We have received physician reports of an additional 101 cases of acute idiopathic pulmonary hemorrhage among infants under 1 year of age. This paper reviews the risk factors for this condition and describes the ongoing surveillance efforts.

KEY WORDS:

hemorrhage, mold, *Stachybotrys*, lung, infant

INTRODUCTION

During 1993 - 1994, ten cases of acute pulmonary hemorrhage and hemosiderosis were diagnosed among infants at Rainbow Babies and Childrens Hospital in Cleveland (CDC, 1994). The affected infants lived in an area of eastern metropolitan Cleveland and East Cleveland. In five infants, acute hemoptysis necessitating readmission to the intensive care unit recurred within 1 day to 6 months of discharge. One infant died. The clustering of the cases in one area of the city, the history of recurrent bleeding, and a history of water leaks or flooding in some of the homes led us to hypothesize that the infants were being exposed to a toxic substance in their homes. Specifically, our a priori hypothesis was that infants with pulmonary hemorrhage

were more likely than a group of comparison infants to live in homes where *Stachybotrys chartarum* was present. (Etzel, 1998)

METHODS

Case-control study

We studied all infants under a year of age who had been admitted to the Rainbow Babies and Childrens Hospital in Cleveland with idiopathic pulmonary hemosiderosis between January 1993 and January 1995. For each case infant, we selected three comparison infants matched by age (within two weeks) who were born in Cleveland or East Cleveland.

We investigated the infants' home environments using bioaerosol sampling methods, with specific attention to *Stachybotrys chartarum*. Air and surface samples were collected from the room in which each infant was reported by his parents or grandparents to have spent the most time.

Fungal propagules were collected on polycarbonate filters using a Gilian pump at a flow rate of 2.0 liters per minute for approximately 1 to 2 hours. Diluted filter fluids were plated on the following media: rose bengal streptomycin agar, cellulose agar, 2% malt extract agar, and dichloran glycerol agar. The plates were incubated at 24 degrees Centigrade for 10 days.

National Surveillance

To determine how common this condition might be, we solicited reports of idiopathic pulmonary hemorrhage in infants under 1 year of age from pediatricians throughout the United States.

RESULTS

Case-control study

Both case and comparison infants lived in homes of an average age of 75-76 years. Many of these homes were built with forced air furnaces with no return air ducts (all return air for the furnace is pulled from the basement rather than cold air return ducts from the living space). Mean colony counts for all fungi averaged 29,227 colony-forming units (CFU)/m^3 in the homes of affected infants (cases) and 707 CFU/m^3 in the homes of comparison infants (controls). The mean concentration of *S. chartarum* in the air was 43 CFU/m^3 in homes of cases and 4 CFU/m^3 in homes of controls. Viable *S. chartarum* was detected in filter cassette samples of the air in the homes of 5 of 9 cases and 4 of 27 controls. The matched odds ratio for a change of 10 units in the mean concentration of *S. chartarum* in the air was 9.83 (95% confidence interval 1.08 - 3 X 10^6). In other words, if there was a 10 CFU/m^3 increase in the concentration of *S. chartarum* in the air the infant breathed, then the infant was 9.83 times more likely to be a case. In surface samples, the mean concentration of *S. chartarum* was 20 x 10^6 CFU/gram and 0.007 x 10^6 CFU/gram in homes of cases and controls, respectively.

To test for interaction with environmental tobacco smoke, a multivariate matched analysis assessed the impact of *S. chartarum* concentration and exposure to environmental tobacco smoke and showed an odds ratio of 21 (95% confidence interval 1.07 - 7.5 x 10^6) for an

increase of 10 units in the mean concentration of *S. chartarum* in the presence of environmental tobacco smoke.

National Surveillance

Since the completion of the 1994 investigation, 27 additional infants in the Cleveland area were diagnosed with acute idiopathic pulmonary hemorrhage. Of the 37 infants, 30 were African-American infants. All of the African-American infants lived in the same geographic area of eastern metropolitan Cleveland and East Cleveland. Twelve deaths with extensive idiopathic pulmonary hemosiderosis have been identified in Cleveland, including seven originally thought to be due to Sudden Infant Death Syndrome (SIDS).

We have received physician reports of an additional 101 cases of acute idiopathic pulmonary hemorrhage among infants in the United States under 1 year of age.

DISCUSSION

The results of our case-control study suggested that infants in Cleveland with acute idiopathic pulmonary hemorrhage were more likely than control infants to live in homes which had *S. chartarum* in the air. We hypothesize that pulmonary hemorrhage occurred when fungal spores or other fragments of the microbe reached the breathing zones of the infants and were inhaled.

S. chartarum requires water-saturated cellulose materials for growth in buildings (Flannigan & Miller 1993). Because of this, it is considered to be uncommon in homes. A study in California found about 3% of 70 homes to have this fungus (Kozak & Gallup, 1985). A study of 52 homes in eastern Canada found *S. chartarum* in one (Miller et al. 1988). A study of 401 homes in southern Ontario, Canada, found it in only three homes (CMHC, 1996).

Stachybotrys species produce trichothecene toxins as well as a series of less toxic phenylspirodrimanes (e.g., stachybotrylactones) (Jarvis, 1995, 1998), a cyclosporin (Sakamoto et al. 1993) and a series of spindihydrobenzofuran-lactams (related to the stachybotrylactams) which are endothelin receptor antagonists (Ogawa et al. 1995; Roggo et al. 1996). The toxicity of *S. chartarum* spores and culture extracts is likely due to a combination of the trichothecenes and other potent immunosuppressive agents (Jarvis et al. 1995, 1998; Johanning, 1996).

In animals, oral exposure to trichothecenes has been associated with gastrointestinal hemorrhaging and anemia (Segal 1983), but an association with pulmonary hemorrhage has not previously been reported in human infants. In mature mice, studies of intranasal administration of *S. chartarum* spores demonstrated severe alveolar and interstitial inflammation with hemorrhagic exudate in the alveoli (Nikulin et al. 1996, 1997). It is possible that very young infants may be unusually susceptible to the toxic effects of trichothecenes because their lungs are growing rapidly, and new vascular capillary formation may be particularly vulnerable to protein synthesis inhibition by these macrocyclic trichothecenes.

Exposure to environmental tobacco smoke appeared to increase the risk for acute pulmonary hemorrhage (Montana et al. 1997). It is possible that this exposure results in areas of pulmonary vasoconstriction with compensatory areas of vasodilation producing increased capil-

lary pressures more likely to hemorrhage. Although no previous relationship with passive smoking has been reported, idiopathic hemosiderosis has been linked to active smoking (Lowry, 1993) in a 15-year old boy.

Idiopathic pulmonary hemosiderosis in children has previously been speculatively linked to indoor exposure to insecticides (Cassimos, 1983). Cassimos reported a case-series of 30 children diagnosed with idiopathic pulmonary hemosiderosis in Greece between 1957 and 1976. The majority of the patients were from families of low socioeconomic status. Eighty percent lived in villages of Northern Greece where their parents were exclusively farmers. Seventy-nine percent of the patients were continuously exposed to stored cereals in their immediate daily environment. The authors proposed environmental factors, perhaps insecticides, as a cause of idiopathic pulmonary hemosiderosis. Perhaps as likely, the grains may have been contaminated with fungi. Mycotoxins on the stored grains may have been associated with these cases of idiopathic pulmonary hemosiderosis. The authors noted the strikingly low incidence of the disease since 1974 in Greece when farmers stopped storing their grain in their homes.

CONCLUSIONS

Infants in Cleveland with pulmonary hemorrhage and hemosiderosis were more likely than control infants to live in homes with toxigenic *S. chartarum* and other molds in the indoor air. The risk appeared to increase when both *S. chartarum* and environmental tobacco smoke were present in the infants' homes.

Surveillance efforts have identified more than a hundred cases of idiopathic pulmonary hemorrhage among infants under 1 year in the United States. The proportion of these cases linked to exposure to toxigenic molds is not known. Additional research is needed to determine whether exposure to toxigenic *S. chartarum* is a risk factor for acute pulmonary hemorrhage and perhaps some sudden infant deaths in other areas of the United States.

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IAQ AND HUMAN TOXICOSIS: EMPIRICAL EVIDENCE AND THEORY

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ABSTRACT

Studies of injury, illness and death occurring in mold-exposed animals and people in the field, observe that the illness called mycotoxicosis results from more complex exposures than can be observed in laboratory experiments with pure mold toxins. Response in field exposures occurs at lower exposure concentrations than those from controlled experiments. Occurrence of signs and symptoms at low exposure levels may result from a number of factors, including a greater spectrum of susceptibility in heterogeneous populations, additive or synergistic actions of multiple toxins, actions of other toxic microbial compounds, allergic and irritant response, as well as concomitant exposure to other contaminants. Changes in the immune system, often reflected as increased susceptibility to infectious illness, are a common finding of low level exposure to toxigenic molds that inhibit protein synthesis. Changes in the immune system are extremely complex, but changes in the endocrine and nervous system accompany them, and may reflect changes in the central neuroendocrine-immune control system. Evaluation of illness of individuals exposed to a combination of irritant, allergenic and toxigenic biological contaminants in wet buildings requires more information about basic effects of individual agents as well as interactive effects of the complex mixtures. In the meantime, case definition for such illness needs to include exposure parameters that actually reflect the nature of the agents capable of effecting illness, rather than some isolated component such as a single mycotoxin. A definition of mycotoxicosis borrowed from agricultural animal and worker exposures, "illness resulting from exposure to one or more toxic molds and their products," could serve. A case definition should also include location of individuals relative to the location of suspect contaminants, time of contact with or exposure to molds and their products, and a list of signs and symptoms gleaned from investigations of known animal or human toxic exposures. Commonality of signs and symptoms in those who show similar time and place exposure parameters could be used to further focus definition of a case. Prudent public health practice recognizes the potency of the toxic agents produced by toxigenic molds, and seeks to protect occupants of buildings once moisture incursion with resultant microbial growth has been discovered.

IAQ AND HUMAN TOXICOSIS: EMPIRICAL EVIDENCE AND THEORY

There is no doubt that humans, other mammals, and birds suffer from mycotoxicoses when exposed to toxigenic molds at high exposures levels. In agricultural exposures, mycotoxicosis

has been defined as an illness resulting from exposure to one or more toxic molds and their products (Akkmeteli, 1977; Joffe, 1978; Mirocha et al. 1976; Hintikka, 1978; Northrop and Kilburn, 1978). Toxicological investigations have elucidated the potency and target tissues of some of the individual toxins via *in vitro* and *in vivo* controlled experiments, as reviewed by Ueno, 1985. LD50 (Lethal Dose50) experiments have established acute single dose lethal levels, that allow crude comparisons of potency of toxic agents, and ranking of susceptibility of animal species by exposure routes. Some mycotoxins, such as aflatoxins and trichothecenes have very high potency. Table I lists LD50s for some trichothecene mycotoxins via exposure routes for three animal species.

TABLE I: LD50 values (mg/kg) of trichothecenes.

Type Trichothecenes	Mouse				Rats				Guinea-pig		
	Iv	ip	Sc	oral	iv	ip	sc	oral	ip	sc	oral
A T-2 toxin		5.2		10.5				5.2			3.06
HT-2toxin		9.0									
Diacetpxyscirpenol (DAS)	12	23.0			1.3	0.75		7.3			
Neosolaniol		14.5									
Monacetoxyscirpenol							0.725				
B Nivalenol (NIV)		7.3	7.4	7.2	38.9						
Fusarenon-X	3.4	3.4	4.2	4.5				4.4	0.5	0.1	
Diacetylnivalenol			9.6								
Deoxynivalenol (DON)		70.0		46.0							
3-Acetyldeoxynivalenol		49.0		34.0							
Trichothecin	300						250				
D Roridin A	1.0										
Verrucarin A	1.5	0.5				0.87					
Verrucarin B	7.0										
Verrucarin J		0.5									

(iv= intravenous; ip = intraperitoneal; sc = subcutaneous)
Adapted from WHO 1990 [after Ueno et al. (1983) and Ryu et al. (1988)]

Such high level exposures cannot be used to determine at what exposure levels clinical signs of illness or injury occur, and LD50s are not useful for assessment of risk to humans from lower level acute or chronic exposures. Acute exposure studies, which determine effect levels and target organs of individual mycotoxins in various animal species, help to elucidate acute effects. Except for cancer studies (van Nieuwenhuize et al. 1997), chronic, low level exposure studies have not been carried out for mycotoxins. *In vitro* work has helped to clarify mechanisms of toxicity for a number of mycotoxins, but not all mycotoxins known, have known effects. New toxins are continually being described, even for well-known species of fungi. For instance, the toxicity of some toxins produced by the genus *Stachybotrys* have been understood for some time, yet Table II shows that new toxins are still being isolated.

TABLE II: *Stachybotrys* toxins.

Satratoxin F	Jarvis et al. 1986
Satratoxin G	Jarvis et al. 1986
Satratoxin H	Jarvis et al. 1986
Roridin L-2	Bloem et al. 1993
Isororridon E	Bloem et al. 1993
Trichoverrols	Bata et al. 1985
Trichoverrins	Bata et al. 1985
Verracarol	Bata et al. 1985
Verrucarin J	Bata et al. 1985
Roridin E	Bata et al. 1985
Cyclosporin FR901459	Sakamoto et al. 1993
Isosatratoxin G	Jarvis et al. 1995
s-Isosatratoxin H	Jarvis et al. 1995
Phenylspirodriamines (9)	Jarvis et al. 1995
Stachybotrylactones	Jarvis et al. 1995
Stachybotrylactams	Jarvis et al. 1995
Water soluble toxins	Nummi & NikPaavola, 1979
Stachybotrydial	Ayer & Miao, 1992

Not all toxic agents produced by this mold have been identified. Jarvis and colleagues (1995) state that less than 10% of the extract from the strains from which they isolated stachybotrylactones and stachybotylactams has been characterized.

In laboratory experiments, the concept of illness or injury thought of as "mycotoxicosis" has come to be attributed to the actions of a specific toxin (i.e., "aflatoxicosis"). In some laboratory work, the exposure agent has been a toxigenic mold or its spores, and the effects are then described as mycotoxicosis (i.e., fusariumtoxicosis and stachybotrystoxicosis). Researchers (Jarvis et al. 1995) have noted that the effects from exposure to isolated toxins are less than the effects from exposure to the mold and its spores. Observations of injury, illness, and death of mold-exposed animals and people in the field, and field experiments in which animals are fed contaminated feed, rather than the mold only, have also shown that such effects occur at lower exposure concentrations than those of single mold, or single toxin exposures in the laboratory.

Exposures of animals in the field differ from controlled laboratory exposures in that animals in the field are of different ages, states of health, and in varying nutritional states. They may be exposed to stressors such as weather, crowding, and competition in addition to exposure to toxic agents. Feeds are often contaminated with more than one mold, and may contain multiple toxins, in addition to other components that may affect health, many of which are unstudied or unknown. The exact nature of the contaminant mix is unknown, as is the concentration and duration of exposure.

In controlled laboratory exposures, animals in exposure groups are of the same gender and age, and are reared under optimum nutritional and environmental conditions. Exposure is to carefully controlled doses or concentrations of a single toxic agent, for a limited, known duration.

Because the results of controlled laboratory experiments appear to give more precise information about toxic effects from mycotoxin exposure, these have often been used as the data base from which to interpret human field exposures to molds, such as those that occur in wet, biologically-contaminated buildings. This practice has led to a seeming dichotomy. Studies of people in such buildings seem to indicate that effects are different from those animals in controlled laboratory experiments, and effects seem to occur at lower exposure concentrations, or from chronic low-level exposure. The seeming dichotomy can be resolved if the more appropriate paradigm for comparison, the animal field study, is used as a source of data for effects and effect levels.

Like animal field studies, human exposures in wet buildings are multifactoral. Contamination often consists of a mixture of bacteria and molds, which can aerosolize and expose people via inhalation and skin contact. Molds and bacteria contain surface and interior molecules that are allergenic. Single toxigenic mold species can produce one or more mycotoxins. Other species in the mixture may also produce toxins in competing for a niche in the building. All molds and bacteria produce (1→3)-β-D-glucans and microbial volatile organic compounds (MVOCs). Dead bacteria can release bacterial endotoxins. In addition there often are other contaminants such as allergens from pets, mites, spiders and cockroaches, and irritant and toxic air pollutants from combustion sources, building materials, and solvents or cleaning materials in the exposure mixture.

Likewise, disease or injury in wet buildings is multifactoral. Exposed individuals differ in levels and duration of exposure relative to their location to the contaminants in the building. They may differ in age, gender, state of health, nutritional status, or genetic predisposition in susceptibility to environmental contaminants. Atopic individuals can develop allergic sensitization to one or more of the organisms, or their effluvia, in the exposure mixture. Some may even develop chronic disease such as asthma or hypersensitivity pneumonitis. Inflammation and effect on mucociliary clearance may increase absorption of other toxic pollutants. Some individuals may develop hypersensitization to organisms or their products. Additive or synergistic effects from exposure to multiple toxic agents can occur. Because biological contaminants from moisture in buildings may remain hidden for long periods of time, and because those exposed often do not complain until serious effects manifest, or they are ignored when they complain, exposure can be of long duration. Because of the fluctuating nature of biological contamination, especially in air, levels of exposure may vary from that measured during an investigation, and may be higher over short periods, especially when molds are blooming.

Mycotoxins have been implicated in illness in wet buildings in a number of recent studies (Croft et al. 1986; Brunekreef et al. 1989; Dales et al. 1991; Verhoef et al. 1995; Johanning et al. 1996; Dales et al. 1998; Etzel et al. 1998, Hodgson et al. 1998). Indications of respiratory illness not attributable to allergy or infection, such as nasal stuffiness, sinus pain, sore throat, wheezing, bleeding from the nose or lung, eye irritation, and severe fatigue, together with the presence of potent toxigenic molds, have led to the hypothesis that mycotoxicoses may be at work. Increased susceptibility to infections, headache, memory and attention deficits, sleep disturbances, nausea, vomiting, skin lesions, and rashes, are among other complaints in wet buildings that have implicated mold toxins. Attempts to reconcile such complaints with the biological action of specific known toxins has not yielded clear dose-response relationships. Such

attempts have ignored the complexity of exposure and response of field exposures that involve populations of mixed susceptibilities and exposure to complex mixtures.

Schiefer (1990) summarized some of the difficulties of diagnosing mycotoxicoses in domestic animals that can easily apply to human mycotoxicoses as well. He writes, "factors that contribute to the difficulty of making a diagnosis: 1) non-specificity of lesions; 2) masking of mycotoxic effects by secondary effects, e.g., through immunosuppression; 3) late appearance of a lesion, e.g., bone marrow damage or neoplasia; 4) interaction of several mycotoxins or presence of other toxicants or deficiency states; 5) species variations in the response to the mycotoxins; 6) difficulty of linking a late appearing effect with a demonstrable cause; 7) low doses of mycotoxins may cause stimulating effects; 8) not being aware of the potential of a mycotoxin as a causative factor as a causative factor in disease."

Animal field studies may present a more useful paradigm for determining exposures and effects of people in biologically contaminated wet buildings. Exposure in such studies is likely to be to multiple agents acting in concert to exert biological effects resulting in complex illness. Effects are more likely to occur at low levels in sensitive neural, hematopoietic or immune tissues, due to the concerted, perhaps additive or synergistic effects of multiple toxic agents. In field studies of farm animals, immune changes from mold exposures that manifest themselves as increased susceptibility to infectious illnesses are observed as Lowest Observed Adverse Effect Levels (LOAELs). Published reports of illness in wet buildings have also reported more frequent and longer lasting infectious illness among those exposed to relatively low levels of mold.

For many mycotoxins that have an immunomodulating effect, such as the trichothecenes, potent inhibition of protein synthesis is thought to be the primary mechanism of toxicity. Inhibition of DNA and RNA synthesis, decreases in enzyme concentration, decrease in immune and phagocyte cell number, and in reduction of functional immunopotent molecules is thought to be a secondary effect of inhibition of protein synthesis. However, trichothecenes also directly affect membrane integrity, increasing fragility of organellar and cell membranes. Deoxynivalinol (DON or vomitoxin) has shown interaction with specific membrane receptors in the central nervous system, causing vomiting. Other trichothecenes such as the tremorgens, affect central and peripheral nerves or have been shown to affect neurotransmitter levels (Cystewski, 1978; Norris et al. 1980; Ciegler et al. 1977) and perhaps to have a role in central nervous system damage (Gordon et al. 1993).

There may not be a single, precisely defined target organ for even a single toxin whose primary effect is on a basic function of all cells, such as protein synthesis. As the organism compensates against damage to multiple tissues, the distinction between direct toxic effect, and compensatory response may blur. Homeostasis is maintained through an integrated neuroendocrine/immune control system that functions through the hypothalamic/hypophyseal axis. Immune cells, as well as neurons, have receptors that respond to neuroendocrine transmitters, while immunological challenges result in changes in catecholamine and corticosteroid hormone levels. Because of these interactions it is likely that injury occurs to the complex control system, rather than "immune," "endocrine," or "neurological" endpoints alone. Since there have been no long-term follow-up studies of patients with signs of immune or neuroendocrine

derangement associated with toxic mold exposure, it is not presently known whether changes that have been documented result in long-term injuries to the centralized control system.

While higher incidence of chronic fatigue and fibromyalgia have been reported anecdotally among those exposed to biocontaminants in wet buildings, it is not known whether these or other potential autoimmune changes can be attributed to such exposure. Auger et al. (1994) report on five cases of chronic fatigue-like syndrome and repeated upper respiratory infections related to airborne mold exposures. Gordon et al. (1993) describe the case of a young man who developed a neurological syndrome that included dementia and a strong movement-induced tremor, putatively from exposure to tremorgenic toxins from *Aspergillus flavus, fumigatus, niger* and *clavatus*, and possibly *Penicillium* and other mold species in silage. Illness began as a week-long flu-like illness followed by tremor and encephalopathy.

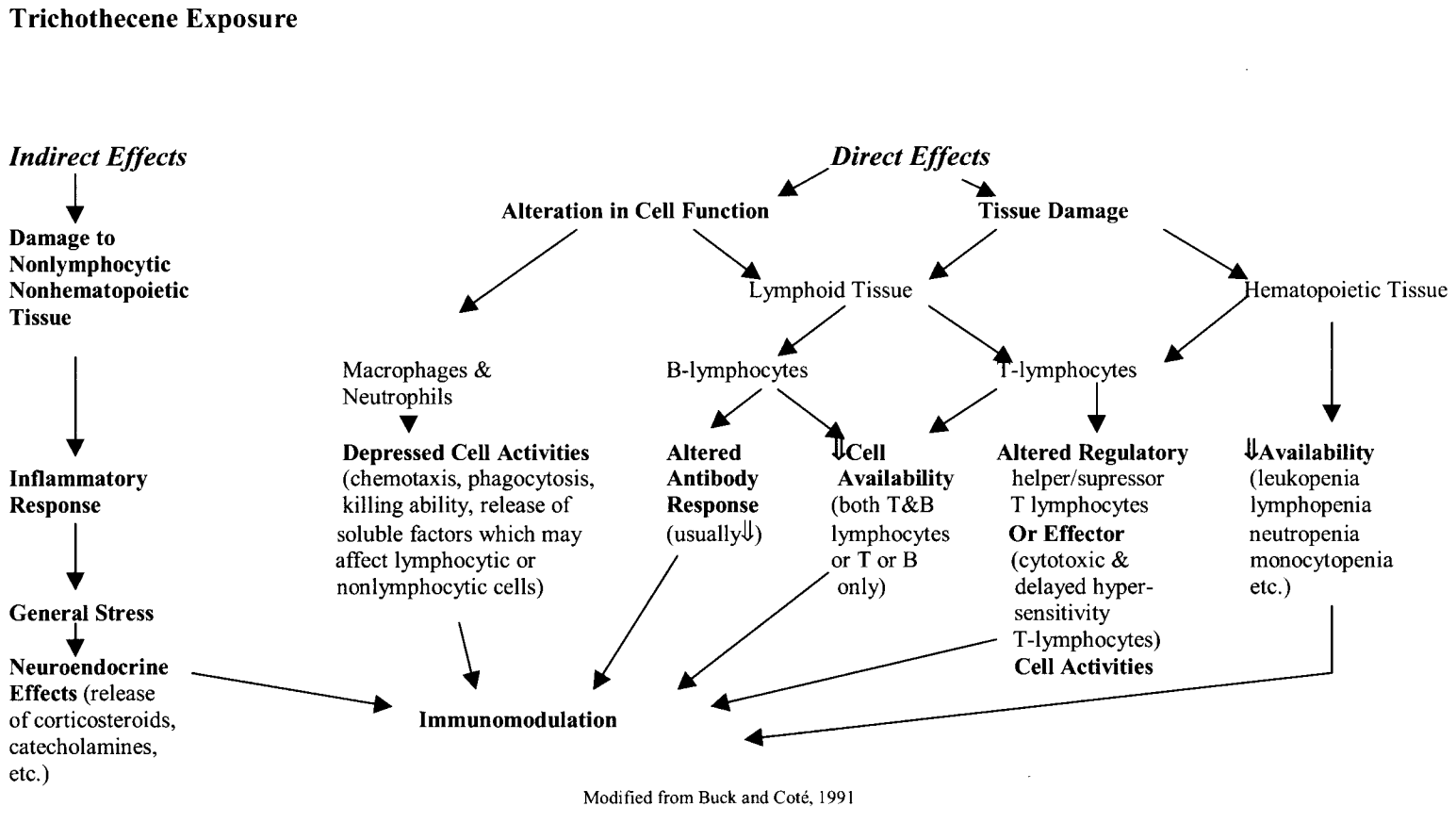
Complexities involved in immunomodulation resulting from trichothecene exposure, summarized from *in vivo* and *in vitro* experiments, are diagrammed in Figure I. It can be seen that the manifestations of illness from trichothecene toxin can be very complex. The diagram does not include other toxic or irritant exposures, such as (1→3)-β-D-glucans, bacterial endotoxins, MVOCs, or other toxic pollutants that could play a role in changing the susceptibility of the neuroendocrine/immune systems.

Evaluation of illness of those exposed to a combination of irritant, allergenic, and toxic biological contaminants in wet buildings requires more information about basic effects of individual agents as well as interactive effects of the complex mixtures. In the meantime, the case definition for such illness needs to include exposure parameters that actually reflect the nature of the agents capable of effecting illness, rather than some isolated component such as a single mycotoxin. The definition of mycotoxicosis in use for decades in describing illness in animals and in workers in agriculture might be a useful starting point: "illness resulting from exposure to one or more toxic molds and their products."

In the absence of specific biomarkers for effect (or even exposure), the presence of toxic molds in air samples, with consideration of all the difficulties of characterizing bioaerosols, should be taken into account. Recognition that "occupants of a wet building" is an insufficient description of an exposure population in a building must also occur. Exposure can differ with proximity to source, or a means of dissemination of contamination from the source, such as an Heating, Ventilating and Air Conditioning (HVAC) system. As much as possible, location of individuals, with and without symptoms, relative to the location of suspect contaminants (i.e., wing, floor, proximity to air distributors if HVAC system is contaminated, or to visible or measured mold) should be described as part of the exposure analysis. Time of contact with or exposure to molds and their products should also be a part of the case definition. A list of signs and symptoms gleaned from investigations of toxic exposures, both from animal observations and known human exposures could be used to focus the definition of a case. Signs and symptoms common to such exposures as demonstrated from dermal or inhalation contact especially, would be useful to classify cases of mycotoxicosis as possible, probable and definite, for epidemiological investigations. Commonality of signs and symptoms in those who show similar time and place exposure parameters could be used to further focus case definitions.

Prudent public health practice recognizes the potency of the toxic agents produced by toxigenic molds, and seeks to protect occupants of buildings once moisture incursion with result-

FIGURE I: Immunomodulation from trichothecene exposure.



Modified from Buck and Coté, 1991

ant microbial growth has been discovered. This may consist of alerting occupants to potential health problems from continuing exposure, or actual removal from exposure through shifting people away from known sources, or removing them from the building if contamination is egregious, and there are health effects confirmed.

When health complaints in buildings with known moisture problems are noted, efforts should be made to determine the number of people affected, and to note the nature of the complaints. This can be done through a survey with an appropriate validated survey instrument, or through individual interview of occupants by a health care provider skilled in taking an environmental exposure history. Location of occupants within the suspect building can be a part of such an inquiry.

A building investigation by a team of professionals, including a building scientist, industrial hygienist, aerobiologist, and a health care provider, together with maintenance engineers or custodians, should follow an investigative plan. Testable hypotheses must be formulated before action is taken. If biological sampling is to be carried out, a sampling plan that is part of a testable hypothesis about location, extent and nature of the contamination, and its potential for exposure of occupants, must be formulated. Likewise, examination of the patient must be a part of the overall investigation, and a thorough exposure history and physical work-up of all who present with health effects, must be a part of the overall investigation.

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COGNITIVE IMPAIRMENT ASSOCIATED WITH EXPOSURE TO TOXIGENIC FUNGI

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ABSTRACT

The objective of the study was to examine whether cognitive impairment was associated with exposure to *Stachybotrys atra*. 20 individuals were examined using a standard neuropsychological battery and a symptom checklist. Results indicate that all individuals seen meet at least 1 of the criteria for a cognitive impairment, with 13 of the 20 (65%) meeting at least 3 of the criteria for cognitive impairment. Preliminary findings suggest that exposure to toxigenic molds are associated with cognitive impairment, specifically deficits in verbal memory, verbal learning, attention/concentration and set shifting. In addition to these cognitive deficits, the sample reported a high number of cognitive, physical and behavioral symptoms. Validation on a larger sample of individuals is indicated.

KEY WORDS:

Cognitive impairment, neurotoxic molds, memory, learning, intelligence, *Stachybotrys atra*

INTRODUCTION

There is an increasing body of literature on cognitive impairment associated with exposure to various forms of neurotoxin (Hartman, 1995; Ziem and McTamney, 1997). None of this research has yet to examine the relationship between exposure of neurotoxic molds and cognitive functioning, which was the focus of the current study.

MATERIALS AND METHODS:

20 individuals who were evaluated (EJ) at the Eastern New York Occupational and Environmental Health Center between January 1993 and July 1998 and who reported cognitive changes following their exposure to toxigenic fungi *Stachybotrys atra* were evaluated for this study. While other neurotoxins may have been present, the details were not provided.

All participants were administered a comprehensive standardized neuropsychological battery (WG). The battery administered included among other tests the Wechsler Adult Intelligence

Scale (WAIS-R), the Wechsler Memory Scale (WMS-R), the California Verbal Learning Test (CVLT), and the Booklet Category Test (BCT). In addition to this battery, each participant was given a symptom checklist. The symptom checklist contained 122 somatic, cognitive and behavioral symptoms.

Data were examined to determine whether or not there were a common set of criteria which would suggest the presence of cognitive impairment. Criteria used to define cognitive impairment were:

1. Verbal I.Q. significantly greater (10 points or higher) than Performance I.Q. on the WAIS-R.
2. Visual Memory Index Score significantly greater (10 points or higher) than the Verbal Memory Index Score on the WMS-R.
3. General Memory Index Score significantly greater (10 points or higher) than the Attention/Concentration Index score on the WMS-R.
4. The total number of responses on the CVLT was at least 1 standard score below the age and gender appropriate mean.
5. Performance on both trial 1 and trial 5 on the CVLT was at least 1 standard score below the age and gender appropriate mean.
6. Impaired performance on the Booklet Category test demonstrated by either inability to complete test or performance in below the 1st percentile.

RESULTS

The sample was primarily female, the average age was 48 years (± 9) and were highly educated, with half the sample having received the equivalent of a Masters Degree or higher (see Table I).

TABLE I: Demographic Characteristics.

Sex:		**Age:**		**Education:**	
Male	5	Mean	48	Mean	17 years
Female	15	SD	9	SD	1 years
		Range	34-66	Range	15-20 years

In order to provide a context for examining the number of symptoms relative to a sample of individuals that were not exposed (n=278), a subset of 44 symptoms were selected that were compatible with a symptom checklist administered to a large group of people with and without disabilities. It was found that the people in the exposed sample reported significantly more symptoms than a group of individuals without a disability (see Table IIa-IId).

TABLES IIA-IID: Frequency Distribution of Responses to Symptom Checklist.

TABLE IIA: Total # of Symptoms out of 44.

	0	1 to 12	>13
Control (mean=2.3; SD=4.5)	52%	44%	4%
Exposed to mold (mean=30.4; SD=6.6)	0%	7%	93%

TABLE IIB: Total # of Physical Symptoms out of 10.

	0	1 to 2	>3
Control (mean=0.4; SD=1.0)	76%	20%	4%
Exposed to mold (mean=7.9; SD=2.0)	0%	18%	82%

TABLE IIC: Total # of Cognitive Symptoms out of 23.

	0	1	2 to 3	>4
Control (mean=1.5; SD=3.0)	61%	14%	12%	13%
Exposed to mold (mean=16.9; SD=4.1)	0%	6%	13%	81%

TABLE IID: Total # of Physical Symptoms out of 11.

	0	1	2	>3
Control (mean=0.6; SD=1.4)	73%	10%	8%	9%
Exposed to mold (mean=5.3; SD=2.7)	11%	6%	6%	77%

The mean scores for both the WAIS-R and the WMS-R (see Table III and Table IV) are in the expected range given the age and education levels of the sample. Based on review of this material, one would be led to believe the group was unimpaired, however with closer examination using the above criteria an impairment becomes evident. Each criteria described above identified between 30%-60% of the sample as having an impairment (see Table V). All the participants met the criteria for an impairment on at least one measure, with 65% of the sample meeting 3 or more of the criteria. (See Table VI)

TABLE III: Mean Scores on WAIS-R.

WAIS-R Test Index Score	Mean Score	Standard Deviation
IQ Verbal	114	13
IQ Performance	109	14
IQ Full	113	14

TABLE IV: Mean Scores on WMS-R.

	WMS-R Mean Score	Standard Deviation
Test Index Score		
Verbal Memory	104	16
Visual Memory	117	15
General Memory	109	16
Attention/Concentration	101	16
Delayed Recall	110	18

TABLE V: Number of Individuals Meeting Each Criteria.

Criteria n	%	impaired
1) Verbal IQ> Performance IQ	10	50%
2)Visual Memory > Verbal Memory	12	60%
3) General Memory>Attention Concentration	9	45%
4) Total responses on CVLT<1 SS below mean	9	45%
5) Trial 1 and Trial 5< 1 SS below mean	6	30%
6) Impaired on Booklet Category	8	40%

TABLE VI: Frequency Distribution of the Criteria Hit.

# criteria filled	#individuals	percent of sample
1	4	20%
2	3	15%
3	9	45%
4	3	15%
5	1	5%

DISCUSSION AND CONCLUSION

In summary, the data presented indicate that individuals with exposure to *Stachybotrys atra* have difficulty integrating visual spatial information, difficulty learning new verbal information, have difficulty paying attention and have difficulty shifting set. When taken together this would suggest the presence of cognitive impairment. These findings strongly suggest that long-term exposure to toxigenic molds results in cognitive impairment in some people. Naturally, these findings require further validation on larger samples. It would be worthwhile to examine if there are mediating factors which differentiate between those with and without cognitive impairment. The extent of impairment should be related to the extent of exposure both in the quantity of toxic substance and duration of exposure. It is also interesting to note that the pattern of cognitive impairment observed herein is quite similar to that reported in individuals who have sustained a minor traumatic brain injury (Dikman et al. 1986).

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SYMPTOMS ASSOCIATED TO WORK IN A WATER DAMAGED SCHOOL BUILDING

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ABSTRACT

This paper describes a cross sectional study of employees from a school, where an annex had a long history of water damage. The annex had infestations by a range of moulds with *Penicillium, Aspergillus, Cladosporium* most often encountered. The employees filled in a questionnaire with questions on sick building syndrome extended with symptoms of toxic alveolitis. After allocating the employees into three groups according to the weekly hours spent in the annex of 0-7 h/week, 8-15h/week and more than 15 hours/week. We found a positive trend for headache, tiredness, nausea and sleeping difficulties with increasing time spent in the annex. This was also true for episodes of fever, shivering or a flue-like feeling. This study emphasizes the need for a standardized way to perform investigations of water damaged buildings and indicates a dose response relationship between exposure to water damaged buildings and symptoms of inflammation and CNS-irritation even in low exposure situations.

KEY WORDS

Moulds, water-damage, school, teachers, fever, CNS-symptoms, dose-response, sick building.

INTRODUCTION

From occupational settings exposure to high concentrations of mould spores have been shown to cause generalized symptoms of toxic alveolitis also known as organic dust toxic syndrome, ODTS (Rylander and Malmberg, 1992). Under these circumstances the fever, flue-like feeling, malaise, headache, nausea, chest tightness, dyspnea and other symptoms most often occur 6 - 9 hours after exposure to extreme concentrations of dust contaminated with mould and bacteria (RaskAndersen and Malmberg, 1990, Sigsgaard et al. 1994b, Sigsgaard et al. 1994a).

Dampness and mould growth has been associated to respiratory symptoms in several studies during the last decades (Martin et al. 1987, Platt et al. 1989, Brunekreef, 1992). Although some studies have pointed towards the fact that recall bias might account for much of the increased risk, a few studies have been able to demonstrate an association between the number of viable

moulds and respiratory symptoms as wheezing or asthma (Platt et al. 1989; Waegemaekers et al. 1989). In a recent cross sectional study inhalation of (1→3)-β-D-glucan was associated with mucus membrane irritation (Rylander, 1998). In this study 6 ng/m^3 of (1→3)-β-D-glucan was associated with an increased prevalence of mucosal symptoms. The present study is a cross sectional investigation of the employees at a school with a history of water damage problems in an annex building for the past 20 years. The teachers complained of a range of symptoms, and wanted the local authorities to take action against the problems.

MATERIAL

THE BUILDING

The school was built in three tempi, the first two in 1942 and again in 1959. This first part is a three-story brick building with a traditional tiled roof. In 1972 a new one-store annex-building was erected next to the old building on top of the small stream crossing the village. The new concrete annex had a flat roof and a relatively large window/wall ratio. Due to flooding from the stream and leakages from the roof during the wet season water was observed from time to time.

To remedy this problem, a new roof was placed on top of the old one in 1993. However this caused the greatest water-damage in the history of the school during the rainy autumn with constant flooding from the leaky roof, until these problems had been solved in the winter of 1993-94. The annex had a mechanical ventilation system which was out of function from 1991 - 94 when it was renovated and reinstalled.

Problems occurred with the wooden floor of the woodwork room. Therefore a polyethylene-membrane was placed on top of the floor, and covered with a new wooden floor in 1989. Just before the current investigation the underlying floor was investigated, and it appeared that it had been decomposed by *Aspergillus versicolor*. During the past years this mould had thrived under the perfect humid conditions beneath the membrane.

THE EMPLOYEES

At the school there was a staff of 43 people during the time of the survey. After two reminders we had a questionnaire back from 36 of these representing 83% of all employees at the time of the investigation.

METHODS

EXAMINATION OF THE BUILDING

The school was technically examined by three of the authors (HJ,EN & MH) during March 1995 where holes were drilled into hot spots of the walls, roofs and floors of the building to get representative samples of moulds from the materials. All hot spots were examined by means of a contact plate with V8 agar. The moulds were grown at 25°C for a week, and identified to genus or species level.

THE QUESTIONNAIRE

Each employee at the school had a questionnaire concerning respiratory and other symptoms that had formerly been associated with exposure to organic dusts. We also included questions on their work at the school and the classrooms in which they were working during the whole week. Due to confidentiality we were not allowed to have identifiers like name, birthday, sex or age on the questionnaire. After coding of each working period we allocated the employees into three groups according to their weekly working hours in the annex building. When we divided the participants according to weekly hours spent in the annex building we found 10 (28%) to be working 0-7 hours per week in the new building, 12 (33%) 8-15 hours, 13 (36%) > 15 hours per week. Only one person (3%) did not reveal the number of hours spent weekly in the new building.

STATISTICS

The variables were analyzed with χ^2 test or Fisher's Exact Test in the two by c tables, and by Mantel Haenszel's test for stratified data, including trend analysis.

RESULTS

We found infestation with moulds in the floor, on the walls and the roof. The moulds encountered were *Penicillium, Aspergillus, Cladosporium, Phoma, Scopulariopsis, Sporothrix, Sporobolomyces* and *Rhodotorula* species dominating in different areas of the building, see table I.

TABLE I: Moulds found in the water damaged school building.

Number of times the mould was found as the dominating species in areas with a severe infestation	**Roof**	**Floor**	**Walls**
Aspergillus spp.	II	II	
Cladosporium spp.	I	I	
Penicillium spp.	III		I
Phoma spp.	I		
Rhodotorula spp.	I		
Scopulariopsis spp.		I	
Sporothrix spp.		I	
Sporobolomyces spp.	I		

We found no difference in the tenure, the contentedness of the current job or the smoking habits among the employees according to the weekly time spent in the water damaged part of the school (WDP). The mucosal symptoms were almost equally distributed among the exposure groups except for sour throat and loss of voice. For these two symptoms we found a significantly positive trend for the prevalence with increasing time spent in the WDP, see table II.

The neuropsycological symptoms were often positively associated with the time spent in the WDP. For headache, tiredness, nausea and sleeping difficulties we all found a positive trend for the symptoms with increasing time spent in WDP, see table III.

TABLE II: Mucosal and skin symptoms among the employees according to time spent in the water-damaged part of the school.

Symptoms at least once a week	Number of weekly hours spent in the damp building					
	0-7 h		8-15 h		16+ h	
	N	%	N	%	N	%
Eye irritation	2	20	3	25	5	38
Nose irritation	2	20	3	25	5	38
Stuffy/running nose	2	20	1	8	4	31
Sinusitis	1	10	2	17	3	23
Sore throat*§	0	0	6	50	5	38
Loss of voice*§	0	0	0	0	3	23
Dry irritated/itchy skin	0	0	2	17	3	23
N	10		12		13	

#: $p < 0.05$ Fishers exact test. §: $p < 0.05$ one-sided trend test.

TABLE III: Neurological symptoms among the employees according to time spent in the water-damaged part of the school.

	Number of weekly hours spent in the damp building					
	0-7 h		8-15 h		16+ h	
	N	%	N	%	N	%
Headache at least once a week*§	0	0	4	33	7	54
Tiredness at least once a week*§	1	10	5	42	6	46
Nausea at least once a week*§	0	0	1	8	4	31
Lack of concentration at least once a week	1	10	1	8	2	15
Difficulties sleeping more than once a month*§	0	0	2	16	4	31
Annoyed without any obvious reason	1	10	1	8	4	31
Out of balance more than once a month*§	0	0	0	0	4	31
N	10		12		13	

#: $p < 0.05$ Fishers exact test. §: $p < 0.05$ one-sided trend test.

Among the inflammatory symptoms shown in table IV we found a positive trend for aches in two or more joints concurrently and for ever having had episodes of fever, shivering or a flue-like feeling not related to a flue or a common cold.

TABLE IV: General inflammatory symptoms among the employees according to time spent in the water-damaged part of the school.

	Number of weekly hours spent in the damp building					
	0-7 h		8-15 h		16+ h	
	N	%	N	%	N	%
Pain in neck or shoulders	2	20	0	0	3	23
Aches in more than one joint at the same time*§	0	0	1	8	4	31
Episodes of fever, shivering or a flue-like feeling not related to a cold or flue*§	0	0	4	33	5	38
Fever episodes during the last 12 months	0	0	2	17	3	23
N	10		12		13	

#: $p < 0.05$ Fishers exact test. §: $p < 0.05$ one-sided trend test.

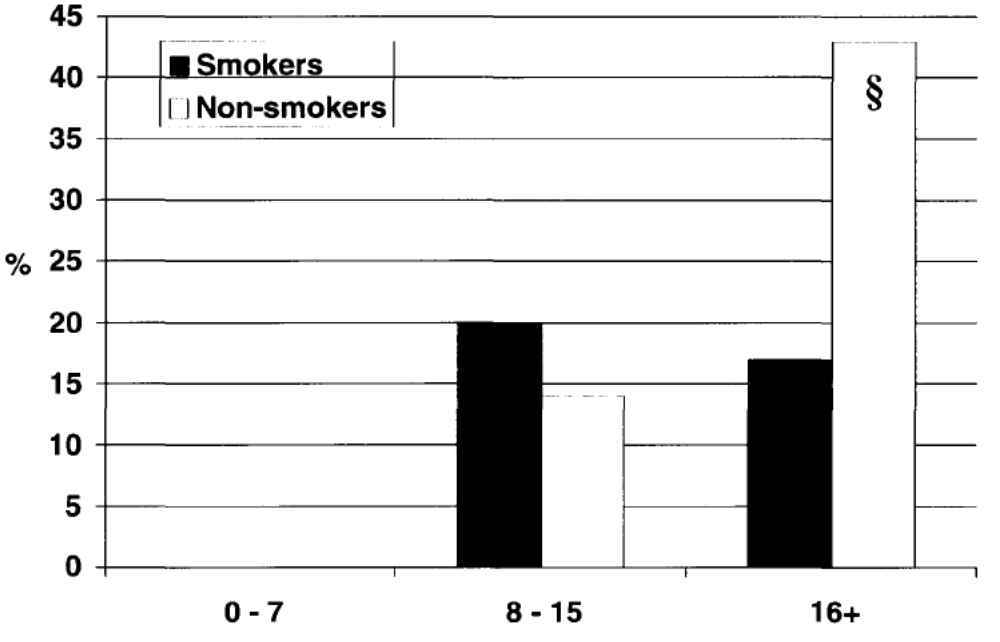
Any cough was positively associated with the time spent in the WDP, see table V. However when we broke the figures down for smoking we found this association to be confined to the non-smokers, see figure I.

TABLE V: Respiratory symptoms among the employees according to time spent in the water-damaged part of the school.

	Number of weekly hours spent in the damp building					
	0-7 h		8-15 h		16+ h	
	N	%	N	%	N	%
Chest tightness	0	0	0	0	1	8
Wheezing	0	0	0	0	0	0
Cough	0	0	1	8	2	15
Cough with phlegm	0	0	1	8	2	15
Any cough*	0	0	2	17	4	31
N	10		12		13	

#: $p < 0.05$ Fishers exact test. §: $p < 0.05$ one-sided trend test.

FIGURE I: The association of cough with the weekly hours spent in the water-damaged part of the school stratified for smoking (smokers and ex-smokers < 12 months collapsed into smokers). §: $p < 0.05$ one-sided trend test.



DISCUSSION

The few demographic variables available did not allow us to perform an in depth analysis of some of the possible confounders such as sex and age on the relation between the hours spent in the WDP and symptoms. However, we did not find any difference in the tenure or the contentedness of the current job between the groups. We included shoulder-neck pain as a controlling variable, and this was not associated with the exposure, table IV. Furthermore the grouping was made post hoc on the basis of the self-reported presence in the WDP. This is relatively easy for teachers who have a schedule that is followed every week independently of our analytical strategy. This gave us a unique opportunity to study the dose response relationship between mould exposure and symptoms without concentration measurements, since there was a great variation of the weekly hours spent in the WDP and we therefore had the opportunity to conduct an analysis with exposure-time rather than concentration as the exposure variable, under the assumption that the exposure were equal all over the annex building and different from the old dry building.

The outcomes we picked for this investigation were the classical symptoms associated with indoor air problems. These symptoms we extended with symptoms of toxic alveolitis as joint ache, fever or a flue-like feeling. Finally we included some questions on neuro-psychological symptoms, since CNS-symptoms occurred after exposure to moulds in some studies (Johanning et al. 1993). Of the classical SBS-symptoms only sore throat and loss of voice were associated with the exposure. It is interesting to note, that the symptoms found in toxic alveolitis seem to occur at increased rates among people exposed to much lower concentrations as found in this study. Cough was more common in the group with most hours spent in the WDP. This increase in cough was found confined to the non-smokers, which is very interesting in the light of the early findings that allergic alveolitis caused by moulds were almost exclusively seen among non-smokers (Terho et al. 1986; Husman et al. 1990; Fink, 1984) and the fact that IgG levels are reduced among smokers exposed to organic dust (Sigsgaard et al. 1992). The neuro-psychological symptoms are the ones which most consistently are associated with the exposure in this study. It has been emphasized in other studies of mould exposed people that moulds might stimulate the neurones leading to imbalances which in turn should lead to the symptoms (Johanning et al. 1993). Another possibility is the fact that especially persons with symptoms might have been under pressure from colleagues and management, because they were the persons most severely affected by the WDP. In a study like the present these possibilities can not be ruled out, and much research is obviously needed in this area, since the people affected are suffering from their occupational environment whatever the causation.

In conclusion this small study shows a dose response relation between the hourly exposure to a water damaged part of a school and symptoms of inflammation and CNS-irritation normally encountered only after high exposures to moulds. This emphasizes the need for a standardized way to perform investigations of buildings where mould growth is expected to be an issue. The study also indicates a dose response relationship between exposure to water damaged buildings and symptoms.

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SENSORY IRRITATION OF MICROBIALLY PRODUCED VOLATILE ORGANIC COMPOUNDS IN MICE DURING REPEATED EXPOSURES

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ABSTRACT

Microbially produced volatile organic compounds (MVOCs) are suspected to cause eye, nose and throat irritation in occupants of moldy buildings. The effect of repeated exposures (30 minutes per day during 4 consecutive days) of mice to 3-octanone (3531 mg/m^3), 1-octen-3-ol (36 mg/m^3), or to a mixture of five MVOCs (58 mg/m^3) via inhalation was studied with a standardized method (ASTM E 981-84). With single MVOCs, no changes in the responses between repetitions of exposure were seen, and only a very slight adaptation in the respiratory response was noted along with the repetition of exposure to a mixture of MVOCs. Thus, during a short-term experiment, repeated exposure to MVOCs did not provoke changes in the sensation of irritation nor cause permanent effects on upper respiratory tract.

KEY WORDS:

microbial volatile metabolites, MVOC, repeated exposure, sensory irritation, indoor air

INTRODUCTION

In mould damaged houses, microbially produced volatile organic compounds (MVOCs), which are perceived as moldy odor, may usually increase VOC levels in indoor air only slightly because of very low concentrations of individual MVOCs. However, MVOCs have been assumed to be partly responsible for the eye, nose and throat irritation symptoms (i.e. sensory irritation) among occupants in moldy buildings (Burge 1990). Especially at low VOC concentrations, irritating responses may even become strengthened with time (Hudnell et al. 1992). The sensory irritation potency of a chemical can be evaluated with an animal model by determining the concentration capable of decreasing the respiratory rate of mice by 50% due to stimulation of nasal trigemial nerve endings by that chemical (ASTM E981-84). There is a relation between the potency of a chemical (or a mixture) determined by the mouse bioassay and sensory irritation response experienced by humans (Schaper 1993).

Since exposure to MVOCs in moldy houses may occur repeatedly, it is important to consider this situation. Repeated exposure to a sensory irritant may exhibit changes in the responses toward this chemical (or mixture). An establishment of adaptation (desensitization), sensitization, or cumulative effects may be detected in mice repeatedly exposed to the same concentra-

tion of a chemical. With repeated exposures, adaptation is revealed with weakening responses time after time, whereas sensitization appears with strengthening responses between exposure periods. If a compound has cumulative effects on the sensory irritation response, a decrease in the respiratory rate of mice during pre-exposure (i.e., control) period of consecutive exposure occasions would be noticed resulting from an incomplete recovery from the previous exposure.

In this paper, we present the sensory irritation effect of repeated exposures to two single MVOCs and a mixture of five MVOCs.

MATERIALS AND METHODS

Chemicals.

The MVOCs studied were 3-octanone, 1-octen-3-ol, and a mixture of 2-methyl-1-propanol, 3-methyl-1-butanol, 1-octen-3-ol, 2-heptanone, and 3-octanone. The relative proportions of 1-octen-3-ol and 2-heptanone in the mixture were 33.4 and 33.3% (w/v) respectively, and the remaining chemicals in the mixture comprised 11.1% each.

The inhalation exposure.

The animal bioassay followed the ASTM E981 (1984) standard. Briefly, four mice were exposed simultaneously in each experiment. The head of the mouse protruded into the exposure chamber while the rest of the body was held in a body plethysmograph sealed from the exposure chamber by a rubber collar around the neck of the mouse. During the experiment, the respiratory signals of each mouse were recorded continuously and collected on a computer. All tests lasted for 1 hour, consisting of 15 min control period when only room air was led into the exposure chamber, 30 min exposure to the tested chemical or MVOC mixture, or room air (i.e., sham exposure), and 15 min recovery period (room air only).

During the 30 min exposure period, the MVOCs were led to evaporate into a glass aerosol generator (Pitt #1) using a constant flow rate and compressed air. The generator was heated when necessary to assist the volatilization of the chemicals. The output of the generator was mixed with room air at the entrance of the exposure chamber (2.3 l). The exposure chamber was ventilated at a rate of 13 - 23 l/min. The concentration of the MVOC (or one of the MVOCs in the mixture) in the exposure chamber was monitored continuously by infrared spectroscopy to verify the continuous and steady concentration. The analyzer was calibrated with known amounts of the MVOC. The concentration was calculated from the amount of the chemical led into the generator and the total airflow in the exposure chamber.

At first, several series of exposures with different concentrations of the test chemicals were performed. A new set of four mice were used in each experiment. This was done to establish concentration-response relationships (i.e., the maximum decrease of the mean respiratory rate of four mice vs the exposure concentration) for the MVOCs, and to determine the potency of the tested chemicals (Korpi et al. 1999).

REPEATED EXPOSURES.

After determining the concentration-response curves for the MVOCs and for the mixture, the repeated exposures with the same set of four mice for each chemical were carried out: The test concentration of 3-octanone was 3531 $\pm$ 21 mg/m^3 which was expected to decrease the respiratory rate of mice by 32% at least during the first exposure period (as predicted from the concentration-response curve established for 3-octanone (Korpi et al. 1999)). The concentration-response curve for 1-octen-3-ol was found to be very steep along with the fact that 1-octen-3-ol was almost 100 times more potent than 3-octanone. In the repeated exposures, the test concentration varied 36 $\pm$ 7 mg/m^3, and the expected decreases in the respiratory rate of mice were in the range of 11 to 20%. The test concentration of MVOC mixture was 57.9 $\pm$ 0.1 mg/m^3 (i.e., the concentration of both 1-octen-3-ol and 2-heptanone was about 19 mg/m^3 and about 6 mg/m^3 of the remaining compounds each). This concentration was calculated to produce a maximum 32 % decrease in respiratory rate during the 30-min exposure period.

The repeated exposure was carried out during four consecutive days, at the same time of the day. No mouse was placed twice at the same position in the exposure apparatus. Control mice (sham exposure) were exposed repeatedly to room air only. Breaths per minute during control period on each day were statistically tested (analysis of variance) to detect any long-term effects on normal breathing frequency.

RESULTS AND DISCUSSION

The decreases in the respiratory frequencies during the first exposure periods were within the 95% confidence limits of the expected decreases. The control experiment with room air only during four consecutive days resulted in 16, 6, 4 and 0% maximum decreases in the respiratory rate of mice, respectively. During the exposure to 3-octanone (3531 mg/m^3) on four consecutive days, the maximum decreases in the respiratory rates were 36, 43, 42 and 30%, respectively (Figure I). This indicates simply some variation around Day 1 curve, which probably can be expected in this bioassay. The low exposure concentration of 1-octen-3-ol (29 to 43 mg/m^3) did not induce the reaction along with the repetitions (the maximum decreases in the respiratory frequencies during the four exposure periods were 8, 13, 5, 4%, respectively). This is an important observation for indoor air quality studies: a concentration of a chemical not likely to cause a significant effect on sensory irritation response did not provoke one after repeated exposures, either. The mean respiratory frequencies during the pre-exposure period on each exposure occasion (to single MVOCs or room air) showed no variance, and, thus, indicating no cumulative effects in mice.

The repeated exposure to the MVOC mixture at a concentration of 58 mg/m^3 produced 37, 30, 29 and 23% maximum decrease in breathing frequence during four consecutive exposures, respectively, (Figure II), indicating a very slight adaptation in mice since the maximum response for the same concentration of MVOCs occurred sooner and was higher on Day 1. Since the recovery from previous exposures was complete (although slower on Day 1), no cumulative effects of the repeated exposures to a MVOC mixture could be detected.

FIGURE I. Mean respiratory frequency of four mice for repeated exposure to 3-octanone at concentration of 3531 mg/m³ during four consecutive days.

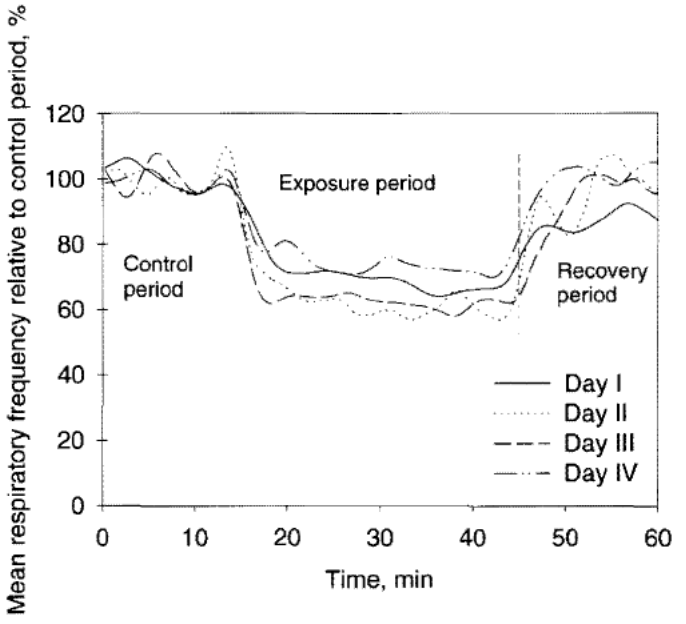
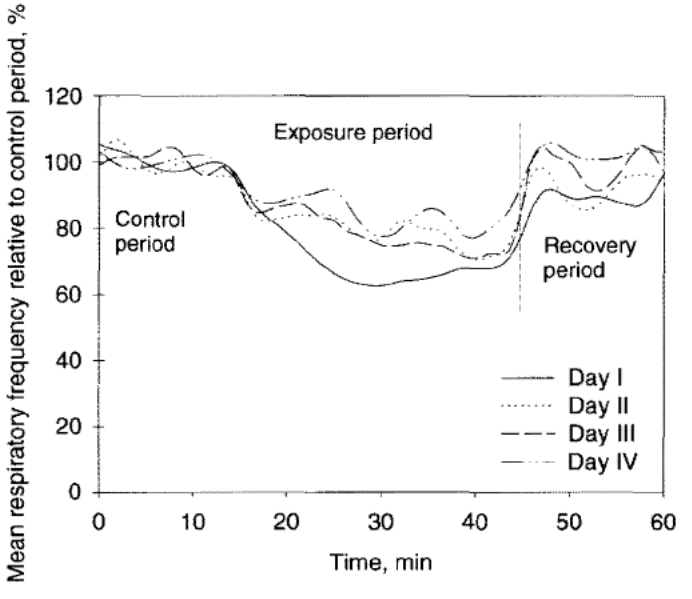


FIGURE II. Mean respiratory frequency of four mice for repeated exposure to a MVOC mixture at concentration of 58 mg/m³ during four consecutive days.



Cometto-Muñiz and Cain (1997) showed that in humans thresholds for nasal pungency, eye irritation and odor tended to decline with increasing number of components in the mixture. In the present study, the concentration of 1-octen-3-ol alone (from 29 to 43 mg/m^3) did not result in a significant decrease in the respiratory rate whereas only 19 mg/m^3 of 1-octen-3-ol in the mixture was enough to decrease the respiratory frequency quite significantly. This suggests that even if concentrations of individual chemicals are below their estimated levels to induce sensory irritation, the combined action of them in a mixture can still provoke irritation of mucus membranes.

Thus, repeated short-term exposure to single MVOCs, a mixture of five MVOCs, or to room air during four consecutive days did not provoke changes in the respiratory responses of mice, and no permanent effects on upper respiratory tract seemed to be caused by repeated exposure to MVOCs. However, due to the short duration of one exposure period and the fact that only sensory irritation effects were studied, conclusions of possible long-term effects of MVOC exposure cannot be drawn. Furthermore, immunological effects were not examined, thus, sensitization and possible inflammatory markers remained unrevealed, even though these may be involved in the consequences of MVOC exposure.

CONCLUSIONS

Repeated 30-min exposure during four consecutive days to MVOCs did not indicate any alteration in responsiveness of mice. The magnitude of changes in breathing patterns was quite similar to those observed following an acute 30-min exposure (Day 1). This suggests that MVOCs appear to act as "pure" sensory irritants and that the effects observed in repeated, short-term exposure are non-cumulative and transient.

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IMMUNOLOGICAL BIOMONITORING IN THE ASSESSMENT OF EXPOSURE TO AIRBORNE FUNGI FROM WASTE HANDLING

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ABSTRACT

The immunologic reaction of workers in different waste treatment facilities to inhalation of mold antigens was studied by determination of specific IgG antibody levels and compared to exposures. Antigens were prepared from cultures of fungi which were dominant in dust samples at these workplaces. In 520 waste workers, 32 patients with symptoms of hypersensitivity pneumonitis (HP), and 98 control subjects, serum concentrations of specific IgG antibodies to antigens of molds (*Aspergillus* and *Penicillium* species) were determined by an indirect immunofluorescence test (IIFT). The highest antibody levels were found in workers in compost plants, followed by employees of garbage sorting facilities. Lower levels were determined in workers at landfills and lowest in collectors of household biowaste. This order reflects the ranking according to the exposure measurements. About 5% of workers had elevated antibody levels, but none complained about typical symptoms of HP. The IIFT proved to be a very sensitive method of antibody detection since even IgG levels of persons subjected to environmental exposure alone (control) could be measured. High exposure to organic dust at workplaces with waste handling is correlated with elevated mold-specific IgG antibody levels.

KEYWORDS

Organic dust, mold exposure, biomonitoring, specific immunoglobulins, *aspergillus*, *penicillium*, hypersensitivity pneumonitis, indirect immunofluorescence technique

INTRODUCTION

Large amounts of airborne molds and actinomycetes occur in organic dust at workplaces where waste is handled [Poulsen et al. 1995]. Antigens of these microorganisms can cause hypersensitivity pneumonitis (HP). Several occupations are known to have an elevated risk for HP, mostly caused by handling contaminated biomaterial in the agro-food sector [Lacey and

Crook 1988]. In addition, cases of HP in compost and other waste workers have been reported [Allmers et al. 1997, Brown et al. 1995, Vincken and Roels 1984, Weber et al. 1993].

In most studies exposure to bioaerosols from organic dust (molds and actinomycetes) is estimated by determining culturable microorganisms (cfu/m³). However, this procedure includes only viable microorganisms, whereas the total count determined by scanning electron microscopy or fluorescence microscopy is much higher [Karlsson and Malmberg 1989]. Because non viable microorganisms and parts thereof also contain relevant amounts of antigens and can contribute to health risks from immunopathogenic effects, our study group has evaluated bioaerosol exposures by measurement of specific IgG antibody formation against antigens of airborne molds in waste workers.

MATERIAL AND METHODS

FUNGI:

In studies of cooperating institutes [Göttlich et al. 1994, Grüner et al. 1998], the frequency of fungal spores in workplaces with waste handling was measured by cultivation methods as colony forming units (cfu/m³) and the different species were identified by standard taxonomic procedures.

Table I: Amounts of fungal spores in the air of German waste treatment workplaces and levels in reference measurements (in cfu/m³ air).

	Fungal spores(in cfu/m³)	
Samples from	**Median**	**Maximum**
composting plants	$>10^6$	$5x10^7$
waste sorting facilities	10^4	10^6
landfills	$<10^4$	10^5
biowaste collecting	$<10^4$	10^5
plant areas (after workshifts)	10^3	10^4
reference areas	$<10^3$	$5x10^3$

Aspergillus and *penicillium* species were the dominant molds in nearly all samples. After taxonomic verification, the following microorganisms - obtained from the University of Stuttgart, Institut für Siedlungswasserbau, Wassergüte- and Abfallwirtschaft - were chosen for antigen preparation and development of the indirect immunofluorescence test (IIFT): *Aspergillus fumigatus, A. nidulans, A. niger, A. sydowi, A. versicolor, Penicillium brevicompactum, P. crustosum. A. sydowi* was identified in several of the investigated plants in low concentrations (<103 cfu/m³). It was used for testing IgG cross reactions.

IIFT:

The IIFT was shown earlier to be a useful method for determination of specific IgG against actinomycetes and molds [Parratt and Peel 1972, Kurup et al. 1977, Zwick et al. 1991]. Antigen preparation and IIFT were performed as follows:

Mycelia and spores of molds were separated from pure cultures grown on malt extract agar plates (Difco Laboratories, Detroit, MI, U.S.A.). The material was rinsed off with a 4% formaldehyde solution into sterile tubes and centrifuged. The supernatant was removed and the pellet resuspended in distilled water. This procedure was repeated thrice. For the IIFT, 20 µl of antigen solution was fixed on specially prepared microscopy slides (bioMérieux, Marcy-l'Etoile,France). Sera were log 4 diluted to concentrations up to 1:1024 and added to the slides, which were incubated for 30 minutes at 37°C. After a washing procedure with phospate buffered saline (PBS), the slide was stained with Evans blue (1%) and specific IgG was detected by a fluorescein labeled goat anti-human IgG (bioMérieux, Marcy-l'Etoile,France). After another incubation for 30 minutes slides were washed again for 10 minutes in PBS. After drying the slide it was mounted with fluoprep (bioMérieux, Marcy-l'Etoile,France) and examined under a microscope equipped for fluorescence viewing (Periplan, Leitz, Wetzlar, Germany).

The highest dilution of each serum which gave definite cell wall fluorescence was taken as the endpoint. Concentrations of 1:1024 were considered positive, and higher dilutions were prepared and investigated in the same way.

In 520 workers of waste treatment plants (composting, n=106; waste sorting by hand, n=213; landfills, n=129; biowaste collecting, n=72) and 98 control subjects (employees in office buildings without humidifiers or air condition), serum concentrations of specific IgG antibodies to antigens of the molds were measured with this IIFT. In addition, specific IgG was determined in 32 patients with symptoms of HP after mold exposure from decayed hay or contaminated humidifiers. Despite lack of exposure data, they were investigated for comparative reasons („positive control"), because none of the waste workers was suspected to be suffering from HP.

Among the workers in composting facilities, 87 answered an occupational, environmental and symptom questionnaire. In addition to mold-specific IgG, antibody levels against 4 actinomycetes were determined using an enzyme linked immunosorbent assay (ELISA) [Stalder and Bünger 1996].

RESULTS AND DISCUSSION

Measurable IgG antibody concentrations were found in over 95% of both the employees and the control group. The highest levels were measured in workers at compost plants, followed by employees of garbage sorting facilities. The lowest levels were determined in workers at landfills and collectors of household biowaste. This order reflects the ranking according to the exposure measurements shown in table I.

TABLE I: Distribution of specific IgG antibody titers against *A. fumigatus* in workers at different worksites, numbers and (percentages).

Source of exposure	IgG-titers	1:1	1:4	1:16	1:64	1:256	1:1024	1:4096
Compost	(n=106)	3	10	17	27	37	10	2
	%	(2.8)	(9,4)	(10,6)	(25,5)	(34,9)	(9,4)	(1,9)
Waste sorting	(n=213)	2	16	52	92	44	7	
	%	(0,9)	(7,5)	(24,4)	(43,2)	(20,7)	(3,3)	
Landfill	(n=129)	5	39	57	21	6	1	
	%	(3.9)	(30.2)	(44.2)	(16.3)	(4.7)	(0.8)	
Biowaste collection	(n=72)	6	26	27	13			
	%	(8.3)	(36.1)	(37.5)	(18.1)			
Control	(n=98)	5	26	35	26	5	1	
	%	(5.1)	(26.5)	(35.7)	(26.5)	(5.1)	(1.0)	
Farming	(n=15)				5	7	3	
	%				(33.3)	(46.7)	(20.0)	
Humidifier	(n=17)		2	6	5	3	1	
	%		(11.8)	(35.3)	(29.4)	(17.6)	(5.9)	

However, for IgG against P. crustosum similar concentrations were found in workers of compost sites, waste sorting plants, and landfills (Table IV). The very low IgG levels in the group of collectors are probably due to the lower median age and shorter duration of employment compared to all other groups. About 5% of workers had one or several (up to five) elevated antibody levels, but no employee complained about symptoms typical for HP. Tables II - IV show the results for 3 different fungi identified in over 95% of all bioaerosols investigated by Göttlich et al. (1994).

TABLE II: Distribution of specific IgG antibody titers against *A. niger* in workers at different worksites, numbers and (percentages).

Source of exposure	IgG-titers	1:1	1:4	1:16	1:64	1:256	1:1024	1:4096
Compost	(n=106)	3	3	21	47	28	4	
	%	(2.8)	(2.8)	(19.8)	(44.3)	(26.4)	(3.8)	
Waste sorting	(n=213)	6	47	95	58	6	1	
	%	(2.8)	(22.1)	(44.6)	(27.2)	(2.8)	(0.5)	
Landfill	(n=129)	1	21	46	53	8		
	%	(0.8)	(16.3)	(35.7)	(41.1)	(6.2)		
Biowaste collection	(n=72)	4	11	34	22	1		
	%	(5.6)	(15,3)	(47,2)	(30.6)	(1.4)		
Control	(n=98)	9	35	40	13	1		
	%	(9.2)	(35.7)	(40.8)	(13.3)	(1.0)		
Farming	(n=15)				6	7	2	
	%				(40.0)	(46.7)	(13.3)	
Humidifier	(n=17)			4	8	5		
	%		(23.5)	(47.1)	(29.4)			

Obviously, there are cross reactions of specific IgG inside the *aspergillus* group, since we found 6 positive (1:1024) titers for *A. sydowi* - a rare fungus identified only in low concentrations - and these positive results were restricted to workers who also had high titers against other *aspergillus* species.

TABLE III: Distribution of specific IgG antibody titers against *P. crustosum* in workers at different worksites, numbers and (percentages).

IgG-titers		1:1	1:4	1:16	1:64	1:256	1:1024	1:4096
Source of exposure								
Compost	(n=106)	8	12	36	41	6	3	
	%	(7.5)	(11.3)	(34.0)	(38.7)	(5.7)	(2.8)	
Waste sorting	(n=213)	4	31	98	63	15	2	
	%	(1.9)	(14.6)	(46.0)	(29.6)	(7.0)	(0.9)	
Landfill	(n=129)	8	14	45	41	16	5	
	%	(6.2)	(10.9)	(34.9)	(31.8)	(12.4)	(3.9)	
Biowaste collection	(n=72)	12	31	21	8			
	%	(16.7)	(43.1)	(29.2)	(11.1)			
Control	(n=98)	6	25	49	18			
	%	(6.1)	(25.5)	(50.0)	(18.4)			
Farming	(n=15)				2	4	9	
	%				(13.3)	(26.7)	(60.0)	
Humidifier	(n=17)		5	6	5	1		
	%		(29.4)	(35.3)	(29.4)	(5.9)		

Elevated specific IgG antibodies to actinomycetes were found in 22 workers at compost plants, 5 of them having also elevated mold-specific IgG titers [Bünger and Stalder 1996]. Ten of the workers had elevated IgG titers solely to molds, resulting in 27 workers with elevated specific IgG to molds and/or actinomycetes of 87 subjects answering the exposure questionnaire.

To compare elevated IgG levels with exposures, the workplaces/activities were divided into two groups either associated with high exposure (compost handling, waste sorting, bunker (delivery), shovel loader driving) or low exposure to organic dust (foremen, maintenance, truck driving, weighbridge).

The correlation between exposure levels and IgG antibody titers was estimated. In the high exposure group, 23 of 49 employees had one or several elevated antibody titers, compared to only 4 of 38 in the low exposure group. This difference was significant.

TABLE IV: Contingency table of exposure versus IgG levels against actinomycetes and/or molds for 87 compost workers answering the questionnaire.

	high exposure	low exposure	total
high titers	**23**	**4**	27
low titers	**26**	**34**	60
total	49	38	87

$p < 0.001$ in Chi-square test

No positive correlation was seen between the duration of employment and the elevated IgG titers. Perhaps this surprising result is due to a healthy worker effect, implying that workers with health problems had given up their occupation prior to investigation or documentation.

CONCLUSIONS

The IIFT proved to be a very sensitive method of antibody detection, since even IgG levels of solely environmentally exposed persons (controls) were measurable. High exposure to organic dust at workplaces with waste handling is correlated to elevated mold-specific IgG antibody levels. Thus these parameters can serve as biomarkers of exposure. The question whether workers with very high antibody levels are at risk for HP will be further investigated by our study group.

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WASTE HANDLING IS STRONGLY ASSOCIATED WITH DYSPNOEA

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ABSTRACT

In a cross-sectional study, we examined the health of the employees in the Danish paper and glass recycling industries. Health was assessed by questionnaire, general health examination, and lung function tests. The exposure to airborne organic dust, moulds, bacteria, and endotoxin was measured by a personal carried sampler during a full working shift.

The predominant result was dyspnoea, reported by 40.7 % in the glass recycling industry against 5.7% in the control group. The measured lung function parameters showed minor but significant decrease in VC and FVC, whereas dynamic values were equal. The dyspnoea was strongly associated with glass handling (OR 14.2, 95%CI: 4.0-50.6), and female sex (OR 4.5, 95%CI: 2.2-9.3). The results were partially explained by variation in the levels of mould, but hardly any other air microbiological air pollution. The presence of 0.5 µm diameter glass particles qualitatively identified at microscopy at the glass recycling plants is an other possible cause.

It is found during the past decade, that people working with sorting and recycling of household waste and paper have an increased prevalence of asthma, chronic bronchitis, and impairment of lung function (Sigsgaard et al. 1994). Airborne organic dust, endotoxins, and exposure to various microbiological parameters have been suggested as possible causative agents.

This study was carried out as part of a program aiming at establishing an evidence based new regulation of the working conditions in the waste handling, sorting and recycling industry. The results of initial surveys of the employees in the recycling industry, and the results of the exposure assessments from the program have been previously reported in details (Midtgaard & Poulsen, 1997).

To our knowledge, so far no studies have investigated the health of workers engaged in sorting and recycling of glass, and this study, therefore had two main purposes: I) to assess the

health of employees working with the recycling of various form of waste with special focus on the recycling of glass, and II) to assess the relation between health characteristics and levels of occupational exposure to a range of bioaerosols and organic dust.

MATERIALS AND METHODS

Twelve plants entered the study: 4 plants handling only glass and bottles, 7 plants handling only or mainly paper and one plant handling household waste for production of compost.

The control group comprised unskilled indoor workers handling mail at two sites in and near Copenhagen. 281 subjects were selected for the study, 214 recycling workers, 67 controls. In the control group 13 persons refused to participate, in the exposed group 14 persons refused. A total of 254 persons were available for analysis: 54 at mail plants, 43 at paper plants, 51 persons at plants sorting both glass and paper, and 106 persons at glass sorting plants. A further description of the industries and the individual plants will be reported elsewhere.

Health assessment

The health assessment included an interview based on a previously completed questionnaire, a clinical examination, clinical lung function tests (spirometry, peak flow monitoring, bronchial challenge), blood samples analyzed for total immunoglobulin E, and nasal lavage analyzed for Interleukine 8 and immunoglobulin A. Health assessment was done not more than one week before or after the exposure measurements.

Exposure assessment

The exposure assessment is described in Wurtz et al. 1998.

RESULTS

Health

The waste handling group was >10 years younger than the exposed group, comprised more females (32% against 15%, $P<0.05$) and had a duration of present employment of 30 months against 90 months in the control group. No significant differences were seen regarding height, weight, smoking habits or weekly working hours.

Respiratory symptoms and lung function parameters are depicted in table I. Dyspnoea when walking fast or slightly uphill is reported by 40.7 % in the waste handling group against 5.7 % in the control group. The waste handling group reported insignificantly more asthma diagnosed by a doctor, more cough and more chronic bronchitis, values not corrected for age or smoking habits. Lung function parameters showed only minor, but significant differences in VC (% predicted) and FVC (% predicted) whereas the other dynamic values were equal. Also diarrhea and nausea was reported slightly more frequently in the waste handling group.

Peak-flow variability was insignificantly smaller in the waste handling group. Except for the dyspnoea symptom, the health pattern showed no significant differences between the 3 waste handling groups, paper, paper and glass, or glass only.

TABLE I: Self-assessed diseases and symptoms, measured lung function, and self-assessed symptoms of the gastrointestinal system among persons working with waste and mail. Values are means (SD) or percentages (n).

	Glass/paper waste (n = 200)	Mail [b] (n = 54)
Respiratory diseases/symptoms		
Dyspnoea when walking fast or slightly uphill, % (n)	40.7% (81)	5.7% (3)***
Asthma (doctor's diagnosis), % (n)	3.0% (6)	0.% (0)
Bronchitis, % (n)	8.5% (17)	3.7% (2)
Cough (at least monthly), % (n)	41% (82)	28.3% (15)
Measured lung function parameters		
% FVC	91.4(13.6)	98.2(16.6)**
% FEV1	91.6(14.1)	95.4(17.9)
% Peak flow	95.2(18.2)	96.4(19.4)
Peak-flow variability referring the individual's own maximum	18.2(8.0)	21.7(10.9)(p = 0.10)
Peak-flow variability referring the individual's own maximum after exclusion of extreme values	16.0(7.5)	19.6(10.4)(p = 0.08)
Gastrointestinal symptoms		
Diarrhea (at least monthly), % (n)	18.5% (37)	13.3% (7)
Nausea (at least monthly), % (n)	13.0% (26)	11.3% (6)

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; - P-value of a Mann-Whitney rank sum test for continuous variables and Chi-square analysis for categorical variables. - a): Those handling paper waste, glass waste or both - b): Those handling mail only.

In table II we show the odds ratio for the characteristics of a person with dyspnoea after adjustment in a multivariate logistic regression analysis. Smoking and weight are included in the

TABLE II -Personal and work related covariates of prevalent dyspnoea. Variables are presented as odds ratios with 95% confidence limits according to strength of association with the outcome after multivariable adjustment in a logistic regression analyses using backward elimination of variables and the maximum likelihood ratio method.

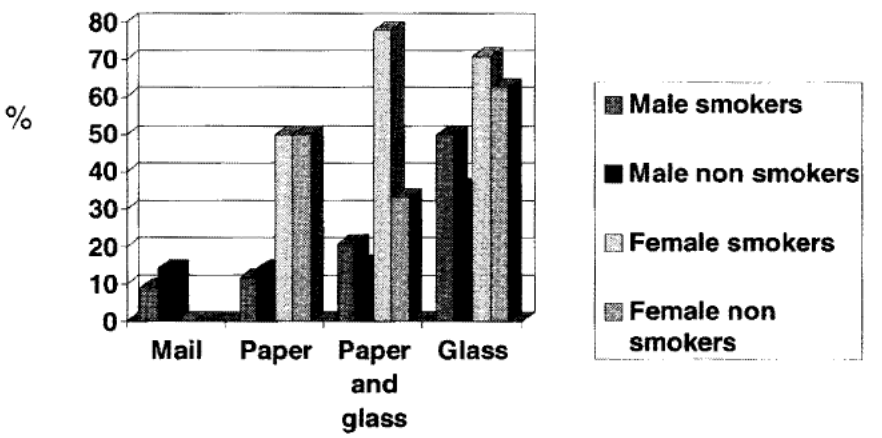
	Odds ratio	95% confidence interval
Female vs male	4.5	(2.2-9.3)***
Works with glass disposal only vs others	14.2	(4.0-50.6)***
Works with glass and paper vs others	5.5	(1.4-21.3)*
Weight, kg ('risk' associated with a one unit change in weight)	1.02	(0.99-1.05)(p = 0.07)
Works with paper waste only vs others	3.4	(0.8-13.7)(p = 0.09)
Smoking vs non smoking	1.7	(0.9-3.3)(p = 0.10)

Excluded from the final model ($p > 0.10$) were: height, previous dust exposure, age, and duration of employment

*: $p < 0.05$; ***: $p < 0.001$

model, but with only insignificant odds ratios. Working with glass alone, glass and other types of waste, or with paper showed the highest odds ratios (3.4 (95%CI:0.8-13.7) to 14.2 (95%CI:4.0-50.6) together with sex, the odds ratio being 4,5 (95%CI:2.2-9.3) for females versus males. Height, previous dust exposure, age and duration of employment were excluded because of p-values >0.10. Figure I shows the crude prevalence of dyspnoea when walking fast or uphill according to type of plant, gender and smoking habit.

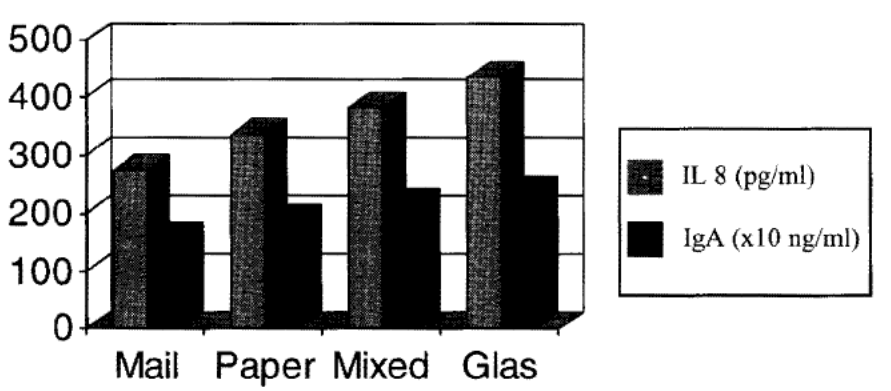
FIGURE I: Prevalence of dyspnoea when walking fast or slightly uphill according to work function, gender, and smoking status.



No significant relation was found to serological parameters. Mean total IgE levels in the paper, glass, mixed, and control groups were 55, 74, 83, and 68 mg/l, respectively. No two groups were significantly different at the 0.05 level, neither in bivariate nor in multivariate analysis.

Results of nasal lavage shown in Figure II.

FIGURE II: Interleukin 8 (pg/ml) and Immunoglobulin A (10 x ng/ml) in nasal lavage. Mean values. Il 8: Spearman R = 0.523, p<0.01; IgA: Spearman R = 0.245, p<0.01.



DISCUSSION

For at least 10 years, the waste recycling industry has been associated with health problems. Health problems previously reported are asthma, symptoms of ODTS, chronic bronchitis, decline in FEV1 and FVC, and diarrhea. Even allergic alveolitis has been suggested as associated with recycling work (Dutkiewicz 1997).

At the first plant for recycling of mixed household waste in Denmark, a majority of the employees developed asthma within the first year of employment (Sigsgaard et al. 1990). Working conditions have improved since due to more restrictive regulations and the general concern about air pollution in workplaces. The levels of air pollution at Danish recycling plants for paper and household waste have decreased during the past decade and now reach levels around or below nationally or internationally suggested levels for total dust and endotoxin and around or slightly above for viable micro-organisms (Wurtz et al. 1998).

The exposure assessment in this study was performed as personal measurements over a work-shift, where the sampling equipment followed the work-site and not the person. This approach was chosen because of intensive job rotation among the workers, with sometimes as little as 30 minutes at each working position. The exposure levels achieved were generally moderate except for the exposure to moulds at manual glass sorting, which equalled that of outdoor waste collection (Sigsgaard et al. 1994, Wurtz et al. 1998). Compared to other studies of waste handling, the average levels of exposure to fungi were relatively high at manual sorting of glass and bottles, whereas the exposure to airborne bacteria and endotoxin was lower (Lacey & Dutkiewicz 1994).

We found job related changes in pulmonary function in the obstructive direction in the exposed group, and nasal IgA and IL-8 were elevated as a possible effect of exposure to fungi.

These results are only weakly influenced by any bias. 90 % of the employees at the recycling plants participated in the study, making selection bias unlikely. Likewise, healthy worker selection would tend to alleviate the associations. Misclassification is minimized as the persons are asked about their number of weekly working hours at each of 5 to 7 different types of work at each plant. Historical information, however, is not included except for duration of employment and previous dust exposure. Classification based on the type of plant includes some low exposed persons in the exposed group. This tends to minimize the health effect and will not lead to overestimation of any job-related risk.

Self-reported dyspnoea associated with waste handling, and particular glass handling, is the main result of the study. The occurrence of dyspnoea associated with waste handling has been indicated in previous studies but has passed rather unnoticed. The frequency found in this study is unexpectedly high. In a normal population only 2-3 % will report dyspnoea when walking fast or slightly uphill, in the control group in this study the frequency was 5.7 %. An overall frequency in the exposed group of 40 % and a frequency among women at glass sorting plants near 60 % could hardly be explained by the moderate levels of microbiological air pollution alone.

Normal confounders are excluded as reasons (smoking, age, weight).

According to the normal levels of total IgE, it seems not to be an effect of allergy but possibly an effect of repeated subclinical incidences of alveolitis. This may have resulted in inflammation, and in minor structural changes in alveolar walls.

A recent case-control study using a self-administered questionnaire (Mullen et al. 1998) found so called idiopathic pulmonary fibrosis to be associated with previous workplace exposure to silica and moulds, but a possible interaction between the two exposures was not addressed. RR for exposure was 16 and 11.1 for silica and mould respectively, but the rate of response to the questionnaire was only 32 %.

The excess rate of dyspnoea at glass sorting plants in the present study may be explained by the presence of fine glass particles in the air. Glass particles were not specifically addressed in the exposure assessment, but numerous glass particles down to 0.5 μm were observed on the dust-filters at the microscopical examination. A study including measurement of DLCO and further evaluation of the exposure to glass dust is planned.

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AN ASSESSMENT OF EXPOSURE AND RESPIRATORY HEALTH AMONG SEWAGE TREATMENT PRESSROOM WORKERS

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ABSTRACT

An exposure and health assessment was carried out to investigate reports of illness among pressroom workers at a large sewage treatment plant. The investigation included environmental monitoring and a self-administered health survey. Environmental monitoring revealed several relevant exposures including predominantly gram-negative bacteria, endotoxin, ammonia, and a volatile organic compound. The health surveys showed a very high prevalence and frequency of a number of respiratory symptoms among workers spending 20% or more of their time in the pressroom. The vast majority reported symptom improvement away from work. The findings suggest that pressroom workers at this plant were experiencing respiratory symptoms as a consequence of workplace exposures. Likely exposures contributing to the symptoms include gram negative bacteria and endotoxin. Ammonia and volatile organics may also have been contributing factors.

KEY WORDS:

respiratory health, sewage industry, waste treatment, sewage treatment workers, endotoxin, gram-negative bacteria, microorganisms, aerosols, organic dusts.

BACKGROUND

In 1994, the Central New York Occupational Health Clinical Center (CNYOHCC) was contacted by management from a large sewage treatment plant and asked to investigate an apparent outbreak of respiratory symptoms among workers in the pressroom of the sludge dewatering facility. Our investigation consisted of a workplace environmental assessment and a health assessment to: determine the type, severity, and prevalence of respiratory symptoms among pressroom workers compared to workers in other parts of the facility; and to identify the type, sources, and extent of exposures in the pressroom potentially contributing to symptoms.

SEWAGE TREATMENT PROCESS

The wastewater from residential, commercial, and manufacturing sources converge in to a single sewage system and is directed to this treatment plant. The treatment process consists of a multi-stage system where solids and grit are removed by physical processes (primary treatment), followed by secondary treatment to remove biodegradable pollutants via bacterial action. Chlorination and chemical additions to neutralize pathogens and to remove some chemical pollutants are accomplished in the tertiary treatment stage, before final discharge.

The resulting sludge is directed to a dewatering operation where it is concentrated by thickeners and polymer addition to facilitate particle sedimentation. Water is then separated from the solids by six large filter presses, which act by applying pressure in a wringer like action to squeeze water out of the sludge. The remaining solid (called filter cake) is moved by conveyor to an adjacent processing and storage facility. Workers access the pressroom on two levels to inspect operations, collect samples, and conduct maintenance / repair work.

METHODS

Exposure Assessment

The following sampling strategy focused on respiratory exposures and was based on preliminary review of work site operations:

*A. Process Related Gases.*Spot and continuous measurements for hydrogen sulfide and ammonia were obtained utilizing area and personal breathing zone samples.

B. Biological Aerosols. Sampling for viable microbes was accomplished using an Anderson single stage (N-6) Impactor. Growth media included 2% malt extract agar (MEA) for fungi; tryptic soy agar (TSA) for bacteria. A sampling time of ten seconds was used to provide readable colony counts

C. Endotoxin. Total aerosol samples were collected of using 5 micron PVC filters and analyzed by the Limulus lysate assay method.

D. Total Volatile Organic Compounds. Summa vacuum canister sampling with subsequent gas chromatography / mass spectrometry was utilized to assess total volatile organic compounds (TVOC) in area air samples.

E. Airborne Mist. A direct reading aerosol monitor was used to survey the work area for airborne particulate/mist levels and to identify generation sources.

The sampling was carried out over a period of seven days, with preliminary work on a single day in late January, and the remaining sampling during the first week of March.

Health Assessment

The health assessment utilized a self-administered questionnaire containing items regarding demographics, job characteristics, symptoms, and medical and smoking history.

RESULTS

Environmental Assessment

Process Related Gases.

Hydrogen Sulfide was not detected in any samples. Ammonia was detected throughout the press room operations. Ammonia concentrations ranged between 5 and 8 parts per million (ppm) during the study period. However, continuous monitoring over 2.5 subsequent days showed ammonia levels rising to 20 ppm. Spot ammonia levels ranged from less than 1ppm in adjacent rooms to 15 ppm near the filter cake. Personal sampling for ammonia showed time weighted average (TWA) exposures of 3.5 ppm for maintenance workers changing belts on the press and 0.5 ppm for a plant operator episodically visiting the pressroom.

Biological Aerosols

Twenty-four (24) samples for bacteria were collected over two test days. Press room bacteria levels ranged from 17,000 to over 85,000 colony forming units per cubic meter of air, (cfu/m^3). The control room and pump room bacteria levels were much lower, ranging from 212 to 424 cfu/m^3. Bacteria levels were greater on the ground level of the press room (Avg. =59,304 cfu/m^3), than the second level (Avg.=41,016 cfu/m^3). Bacteria levels were greater at the west wall (exhaust fan side) then at the east wall (air supply side).

Bacteria identified in all samples included the following (in descending rank order): gram-negative bacteria (ranging from 53-85% of the total); *Micrococcus; Nocardia; Staphylococcus; Bacillus; Rhodococcus; Actinomycetes.* No growth of thermophilic actinomycetes was detected.

Samples analyzed for fungi showed growth ranging from 7 to 212 cfu/m^3 in the press room. Geotrichum was the most common fungi detected. Field control samples for bacteria and fungi indicated no growth

Endotoxin

Ten air samples were collected for airborne endotoxin levels; eight in the press room and two in the background areas. Endotoxin levels ranged form 13.2 to 48.8 nanograms per cubic meter (ng/m^3) in the press room. Endotoxin levels in the control and pump rooms were 0.29 & 0.08 ng/m^3, respectively. Endotoxin levels varied by location in a distribution similar to bacterial levels, with areas nearest the mist sources having the highest levels. Five of the eight press room samples were greater than 20 ng/m^3.

Total Volatile Organic Compounds

Twenty VOCðs were detected at concentrations less than 12 ppb. A compound with a C9-C14 hydrocarbon matrix was detected at levels at least two orders of magnitude greater than the other twenty. The petroleum distillate (kerosene) component of the polyacrylamide polymer used in the dewatering process was the likely source of this substance.

Airborne Mist

Water mist was visible at the lower sections of each operating filter press. Mist concentrations were highest near the discharge pipes and cascading water on the filter presses (5.0 to 9.0 milligrams per cubic meter of air (mg/m^3)). Generally, mist concentrations were highest on the ground floor, with typical levels between 4.5 to 6.0 mg/m^3, compared to typical levels between

1.5 to 2.5 mg/m^3 on the upper level. Mist concentrations increased from the east to west wall. Background aerosol levels were less than 0.10 mg/m^3 in the control room, pump rooms, and outside the building.

HEALTH ASSESSMENT

Response Rate, Exposure Groups, and Demographics

Questionnaires were distributed to ninety-seven (97) employees from four departments. Seventy employees responded (72%). The response rate was similar from all departments. Sixty eight respondents were male. Respondents were divided into two exposure groups based on self reported time in the press room. Fifty-two individuals were classified as low exposure (0-19% of their time in the press room) and seventeen as high exposure (20% or more of their time in the press room). The age distribution of respondents from both exposure groups was similar.

Prevalence, Severity, and Time Course of Symptoms

Among the high exposure (HE) group, there was a very high prevalence of a number of symptoms including nasal congestion (94%), irritated nose (94%), cough (82%), throat irritation (76%), and headache (76%). Irritated eyes (47%), chest tightness (35%), wheeze (41%), and shortness of breath (24%) also occurred frequently among the high exposure group. Almost all symptoms were less prevalent in the LE group with the differences reaching statistical significance for throat irritation, cough, nasal congestion, and irritated nose. Symptoms were also consistently reported as more frequent in the HE group with statistical significance reached for nasal congestion, irritated nose, throat irritation, cough, headache, and wheezing.

Two thirds to three quarters of the individuals in both exposure groups noted improvement of symptoms after work and on weekends. Sixty-eight percent of individuals in the LE group and 89% of individuals in the HE group noted symptom improvement on vacation. This difference was statistically significant. Workers in the HE group were significantly more likely to report that their symptoms had improved since an exposure control intervention had taken place in the press room.

The proportion of current and ex-smokers was slightly higher in the high exposure group, but the difference was not statistically significant.

DISCUSSION

This study strongly suggests that sewage treatment pressroom workers experience respiratory symptoms likely to be work related. Environmental data from the plant suggests several sources of exposure that may be contributing to the symptoms.

Pressroom workers reported a very high prevalence of upper respiratory symptoms and a significant prevalence of lower respiratory symptoms (such as wheeze and chest tightness). Almost three quarters observed an improvement of symptoms away from work, with almost ninety percent improving on vacation. In addition many reported decreased symptoms following a workplace intervention to reduce mist exposure. Pressroom workers reported symptoms consistently more frequently then non-pressroom workers. Despite the relatively small size of

the pressroom group these differences reached statistical significance for a number of symptoms.

The exposure source contributing most significantly to symptoms in the press room is likely the high level of bacteria and endotoxin identified in aerosolized mist coming off the presses. Symptoms noted by individuals at the plant are consistent with published descriptions of workers exposed to similar materials (Rylander R. 1994a; Zuskin E et al. 1993; Rylander R. 1994b; Melbostad E et al. 1994).

Microorganisms originating in the sludge were dispersed and transported in the pressroom by fine water droplets (mist) generated by agitation, sprays, and cascading water. Mist and airborne bacteria levels in the press room were much greater than background levels (control & pump rooms). Gram negative bacteria were found to be the predominant organism in the samples.

Endotoxin was found at levels which were consistent with bacteria levels measured in the press room (i.e. the highest levels of endotoxin were identified in the areas with the most bacteria). Mucous membrane irritation and changes in pulmonary function have been reported at levels of endotoxin below those found in the press room (Olenchock SA. 1990).

During the two testing days ammonia gas levels were relatively low (less than 8 PPM), but increased to 20 PPM during the next two and one-half days of continuous monitoring. This suggests that ammonia gas concentrations could approach or exceed occupational health limits potentially affecting health on an episodic basis in the filter press room. Workers described episodes of high ammonia odor on 'bad days' which supports this notion. Filter press cake appeared to be a primary source of ammonia gas. However, increased ammonia gas concentration over the weekend occurred during the operation of a sludge treatment process in an adjacent work area, suggesting the contribution of a non pressroom exposure source.

The C9-C14 hydrocarbon matrix, attributable to the kerosene component of the polymer mixture added to the sludge stream may have been a minor factor contributing to symptoms (Molhave L 1992).

SUMMARY

Taken together, the high prevalence of many symptoms among the high exposure group, the improvement of symptoms away from work, the nature of the symptoms, and the similarity of the two exposure groups with regard to potentially confounding factors, provides good evidence that individuals who spent more time in the press room experienced respiratory symptoms due to exposures originating in that environment. The nature, frequency, and severity of the symptoms suggest a problem of significant health impact, including the possible development of asthma.

The exposure assessment identified several airborne agents which pose a risk of respiratory effects to exposed workers. In particular, *bacteria* and *endotoxin* associated with gram negative bacteria were found at levels which have been associated with adverse respiratory effects. In addition, the levels of ammonia and volatile organic compounds found in this evaluation can contribute to respiratory irritation. This multiple exposure scenario involving chemical irritants and bacteria presents a situation where respiratory effects could be expected.

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CHRONIC TOXIC ENCEPHALOPATHIES APPARENTLY RELATED TO EXPOSURE TO TOXIGENIC FUNGI.

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ABSTRACT

This report concerns four cases of neurotoxic encephalopathies possibly caused by a prolonged exposure to toxin producing filamentous fungi (molds) in the workplace. We will also demonstrate that complete fungal evaluation with species identification and complementary cytotoxicity testing can be useful to identify possible causes of health effects of mold exposures.

KEY WORDS :

toxic encephalopathy, toxigenic fungi, molds, mycotoxins, *Aspergillus fumigatus, Penicillium crustosum, Penicillium brevicompactum, Fusarium incarnatum, Penicillium aurantiogriseum,* biological contamination.

INTRODUCTION

In 1987, a report by Tobin et al. (1987) published in Canada, drew for the first time, the attention of public health officers to the danger of exposure to spore-borne toxins in indoor air. Fungal toxins have irritant, immunosuppressive, neuro-toxicological and carcinogenic effects (Hendry et al. 1993; IARC, 1993). These include damage to the central nervous system (CNS), immune functions, gastro-intestinal tract, the heart, liver and kidney (Beardall and Miller 1994; Prelusky et al. 1994). The American Industrial Hygiene Association (AIHA) also affirmed the risks of mycotoxins in their proposed guidelines for bioaerosols assessment (Dillon et al. 1996).

Recent reports have suggested neurotoxic symptoms in human subsequent to exposure to airborne spores of toxigenic fungi (Auger et al.1994; Croft et al. 1986; Johanning 1996; Recco et al. 1986).We report four cases who were diagnosed with type 2b solvent type syndrome (Lundbert et al. 1995) with similar neuropsychological findings concurrent with sensitive neu-

ropathy and asthma in some of them. No other cause was discovered except the exposure to airborne toxigenic fungi in the workplace.

MATERIALS AND METHODS

Building history

1-First building: It is a fifty year old harbour station. In 1985, a waiting room was built over a crawl space. At the same period, the basement was excavated and repaired. In 1992, there was a sewer back up in the building, and appropriate cleaning measures were taken. There were also episodes of water leaking from the roof which was repaired. The building has one floor over a basement and for one third of the surface a crawl space. On the first floor, there are two offices and a meeting room, both carpeted. There is also two washrooms, and waiting room and a photocopier. Crawl space odor was discernable in the offices. The basement is constructed from concrete with gypsum wall. Visual inspection revealed numerous water leaks and mold growth. There was a strong musty odor. Smoke tube testing demonstrated that the building was under negative pressure to the crawl space allowing the crawl space air to be spread to other areas of the building.The relative humidity was often over 80% in the crawl space and varied between 25-65% in the rest of the building depending on the season the measurements were made. Formaldehyde and mineral spirit were undetectable in the workplace air. CO2 readings were in appropriate range (350 ppm outdoors, 400-500 ppm indoors). This harbour is located very far from any environmental source contamination. The potable water was from commercial bottling suppliers and was brought with the occupants when they move to a second office where their symptoms began to resign.

2-Second building: This medical office was located in a basement of a twenty years=old building. It suffered severe flooding in March 1992. On June 14, 1993, the clinic was inspected by a public health officer. He was able to detect a strong musty odor, traces of water damage and rotting wood frames. There was also a sumppump located into a slimy well. The premises were poorly ventilated. This building is located in a plushy suburban city and there was no obvious environmental source contamination around. The occupants consumed water from the municipal water supply aqueduct which was devoid of problem at the time.

Microbial samples

1-First building: Tests for airborne bacteria and fungi were made on two occasions; once in the winter and in the spring, after a thorough cleaning of the building. Samples were obtained using the two stage Anderson N6 sampler (Andersen Instrument Inc, Atlanta, GA, USA). The sampling rate and time were 28.3 L/min. for two minutes. In the crawl space, sampling time was shortened to one minute because of the higher concentration of propagules. The airflow was calibrated with a Kurz heating wire flow rate calibrator model 543 (Kurtz Instruments Inc, Carmel Valley, CA, USA). The media used were Sabouraud Dextrose Agar (SDA) for fungi and Trypticase Soya Agar (TSA) for bacteria. Dust samples were collected with a vacuum cleaner and a surface air system (SAS) model 5203 (Pool Bioanalysis Italiana, Milan, Italy) at a flow rate of 180 l/min. for one minute. These methods allow the detection of fungal problems not easily found by air sampling methods (Dillon et al. 1996). The attachments were cleaned with 70%

ethanol. To minimize cross contamination, dust sampling was performed first in the homes of the cases and then in the problem building. Samples of dust were also collected with toilet paper. For fungal analysis, a portion of the sample was weighed and was diluted to extinction in sterile water and cultured by the spread-plate method in 2% with rose bengal and a 2% MEA with 10% NaCl malt extract agar (Miller et al. 1988).

2-Second building: scraping and dust samples were collected and cultured as previously by spread plate method with Rose-Bengal, cellulose and Czapek (Miller et al. 1988, Dillon et al. 1996)).

Cytotoxicity testing

The remaining portions of the dust samples were weighed and extracted with acetonitrile water and the extract defatted with hexane. The extracts were dried and redissolved in cell culture medium and diluted and a 100x dilution series prepared. Samples were incubated with swine kidney (SK) cells to which the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazole-2-yl]-2-5 diphenyltetrazolium bromide) had been added. Optical density was measured spectrophotometrically at 510 nm. Viability of the cells was determined by the extent of conversion by mitochondrial enzymes to purple formazan crystals. Measurements were made of the minimum concentration of extracts that caused unambiguous cytotoxicity (80% decrease of cleavage activity in MTT as compared to control cells (Gareis, 1994).

Medical evaluation

A preliminary physical examination was done by one physician (PLA), followed by a thorough neurological assessment by a certified neurologist (RB). They were finally seen by a certified neuropsychologist (JD) for detailed neuropsychological evaluation. This latter examination included a series of tests designed to measure intellectual abilities (Wechsler Adult Intelligence Scale, revised WAIS-R), memory functions (Wechsler memory scale, revised Rey complex figures, Rey Auditory Verbal learning test, Recognition test of non familiar faces), language functions (Token test, Wingfield Naming test, Chapman-Cook Reading test), attentional and concentration capacities (Wisconsin card sorting test and word fluency test), visuospatial and visuoconstructive abilities (Mooney faces test and Hooper) as well as coordination capacities (Purdue Pegboard Test and Substitution subtests of the WAIS-R) and sensorimotor functions (two-points discrimination test, passive movements and double stimulation on the hand, body and face). It is important to note that norms of each test were used to determine whether the performance on a particular task was significantly impaired or not (Lesak, 1983).

RESULTS

Exposure data

The results of sampling for airborne bacteria and molds are presented in Table I. Data from the sampling taken before and after cleaning showed the presence of high numbers of gram positive bacteria. The sample taken before the cleaning shows a distribution of species in the crawl space and basement very different than that from outdoor air. Species identified in base-

ment and crawl space air samples included *Aspergillus versicolor, A. fumigatus* and *Penicillium auran-tiogriseum.*

In the medical clinic, species identified included *Penicillium crustosum, Penicillium brevicompactum, Fusarium incarnatum.*

TABLE I: Concentration (CFU/m³) of bacteria and molds in the workplace in the first building.

Location	Crawl space	Basement	Office	Outdoors
94-01-10				
bacteria	710	590	210	ND
gm-	40	40	ND	ND
gm+	680	550	210	ND
95-05-11				
bacteria	TNTC	TNTC	650	110
gm-	TNTC	TNTC	650	70
gm+	TNTC	TNTC	ND	40
94-01-10				
Acremonium	790	1375	160	ND
Aspergillus sp.	210	250		
Penicillium sp.	110	460		
Cladosporium	40	60	40	210
95-05-11				
non sporulating	1206	0	200	0
Chaetomium	0	610	ND	ND
Aspergillus sp.	140	450	40	ND
Cladosporium	20	ND	40	3280
Vertilcillium	20	ND	ND	ND
unknown	ND	70	ND	20
Penicillium	ND	20	ND	ND
Monilia	ND	20	ND	ND

ND: Not detected
TNTC: Too numerous to count

Data on the cytoxicity are presented in table II. Dust retrieved from the crawl space and basement floors as well produced cytotoxic extracts. Dust wiped from the floor of the office space after cleaning produced extracts 10-100 times less cytotoxic (data not shown).

TABLE II: Cytoxicity of extracts of dust samples to swine kidney cells.

sample location	cytoxicity (mg/ml)
crawl space	13
basement	3
office	0.8
control 1	0.4
control 2	0.2

Results are expressed as the reciprocal x 100 of the minimum concentration that caused unambiguous cytotoxicity (mg extract/ml culture media; higher values the higher the toxicity)

MEDICAL FINDINGS

1. The first patient was a forty two year old man who gradually noticed two months after arriving in the building (1985) symptoms including: lassitude, difficulty in mental concentration, extreme fatigue, memory problems, insomnia, palpitations, muscle soreness, dizziness, irritability, nervousness, diminution of libido, dysgeusia, alopecia, tingling in hands and feet, clammy hands. He stopped smoking in 1985, is a social drinker and his medical history is clean except for mild allergic reactions to weeds, feathers, and cat dander. Physical examination was unremarkable except for a socklike pattern of sensory diminution of skin pain and temperature sensation. This was later confirmed by electromyographic study in the lower limbs. Biological testing comprising hematological, renal, liver, thyroid function tests and blood lead, mercury and arsenic were all within the normal ranges. This man had also been directed to a specialized laboratory for investigation of sleep apnea. Nocturnal oxymetry failed to confirm this diagnosis.Serum precipitins for *Aspergillus fumigatus* were absent. At first this man did not make any relationship of his disease with his occupational environment but later reported an improvement when he left the building for more than a week.
2. The second patient was a thirty four year old woman who noticed 7-8 months after her arrival in the building (1985) symptoms of fatigue, irritability, headaches, muscle soreness and weakness, insomnia, dysgeusia, mild alopecia, repetitive sinusitis, problems of memory and mental concentration and finger tingling. She had noted an unusual lower level of productivity at work. Six years later (1991) she started to complain of wheezing, cough, and shortness of breath; the diagnosis of asthma was made and she was then treated with bronchodilators and corticosteroids. Her medical past was also clear with no evidence of atopy in her family. She was a smoker.Physical examination was unremarkable except for suspected clinical bilateral carpal tunnel and thoracic outlet syndromes that could explain the finger tingling sensations. Biological testing comprising hematological, renal, liver, thyroid function tests were all normal. Immunology testing yielded slightly elevated total IgM with normal IgE and normal complement levels.
3. The third patient was a 54 year old pediatrician who had been working in that clinic located in the second building for a short period. He commenced to complain of fatigue, memory and mental concentration difficulties at the beginning of september 1992. His condition worsened while still working in this environment. In may 1993, a diagnosis of chronic interstitial nephritis was made. The disease was consecutive to light chains deposits. Even though his renal disease progressed, he felt a lot better when he stopped working in his office. He finally died five years later from renal failure.
4. The fourth patient was the 46 year old pediatrician's medical secretory who also began noticing fatigue, memory and mental concentration difficulties early in may 1992. Her condition worsened to the point that she had to resign from her job. On cessation of exposure she began to feel better. But she never felt as good as she was before working in that environment.Today, she feels easily tired with attention problems. On physical examination, the neurologist could elicit signs of a sensitive neuropathy which was not confirmed by nerve conduction studies. The biological work-up was essentially normal.

NEUROPSYCHOLOGICAL TESTING

The first case performed within normal limits for the majority of the tests, but showed significant deficits in tests measuring visual-spatial abilities and memory functions with difficulties in general memory and attention (Three months after withdrawal from the building). An identical series of tests were run one year later (fifteen months after withdrawal from the building) and his capacities had improved. At this point in time, he showed improvement in almost all of the tests except for an attention deficit which we think is permanent. His symptomatology has improved markedly but, while not feeling constantly fatigued as before, he now gets easily tired on efforts demanding mental or physical stamina.

Similar findings were retrieved in the second case after the first batch of tests i.e., anomalies with visual memory, attention difficulties and mild decrease in cognitive functions (Three months after withdrawal). Similar tests were performed one year later (Fifteen months after withdrawal) and were normal. No cognitive deficit was detected during the second assessment. Her neuropsychological symptoms had improved substantially but she was still experiencing asthmatic bouts even though she had stopped her medication on a daily basis.

The third and the fourth cases showed similar findings but on more intense scale. The medical secretary remained the most severely affected. Abnormal results were found in tests measuring her verbal comprehension, her capacities for problem resolution and her adaptation to changes in instruction. Two years later, the physician had improved as regard his visual abilities even though his renal condition was worsening, whereas for the female the neuropsychologic findings remained identical.

DISCUSSION

These four workers presented with the necessary criteria of neurotoxic encephalopathies (Lundbert et al. 1994). They definitely improved after fifteen months of leaving the workplace except for one. There was no intrinsic organic cause for the appearance of these syndromes. Likewise no other neurotoxic environmental agents (heavy metals, solvents) were detected or suspected in significant probability to be accountable for the clinical findings. The only abnormal circumstance was the high exposure to a number of toxigenic molds in the workplace. Data on the cytotoxicity are presented in table II. Dust wiped from the crawl space and basement floors of the first building produced cytotoxic extracts. Dust wiped from the floor of the office space after cleaning produced extracts were 10-100 times less cytotoxic

At this point in time, it could be argued that the clinical improvement in the the neuropsychological testing was from training effect of repeating the same tests instead of cessation of exposure to these toxic molds. We think that this is not the case for two reasons: firstly, the delay between the two testing sessions were fairly apart so that it would be difficult for the patients to remember many of the very specific and complex details of the test items, and secondly equivalent but different version of the tests were administered in the second evaluation.

Species identified included highly toxigenic species *Aspergillus versicolor* the toxigenic and pathogenic mold *A. fumigatus* and the toxigenic molds *Penicillium aurantiogriseum, Penicillium crustosum, Penicillium brevicompactum, Fusarium incarnatum.* All these can produce in some ways or another

neurotoxic metabolites (Tobin et al. 1987, Samson et al. 1995).Also in an occupational setting, an acute CNS disease with tremor was related to an exposure coming from a cloud of organic dust released from a silo highly contaminated by *Aspergillus* spores (Gordon et al. 1993).

A recent article (Hodgson et al. 1998) has described neuropsychiatric symptoms in occupants working in a building contaminated by two toxigenic fungi: *Stachybotrys chartarum* and *Aspergillus versicolor*. The neuropsychological tests were identical to a control population. To explain the discrepancy between our results with theirs, we think that the battery of tests we used did cover a wider range of cognitive functions. The other possibility could be that the quantity of the three trichothecenes detected in their building was insufficient to produced irreversible CNS damage.

CONCLUSION

We have described four cases of encephalopathies apparently related to exposure to toxigenic molds. The presence of toxicogenic molds in an environment suggests that when two or more occupants of a dwelling suffer from neuropsychological symptoms, a detailed exposure analysis should be done. Unfortunately we were not able, because of financial and logistical constraints, to search for mycotoxins in the environment and relate them with their presence in some biological fluids from the patients. This would have certainly brought an higher level of proof. In the meantime, we would suggest that these CNS complications to mold exposure exist and that these findings should be confirmed by other case reports and rigorous epidemiological studies.

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INHALATION OF (1→3)-β-D-GLUCAN IN HUMANS

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ABSTRACT

Fourteen persons living in houses with levels of airborne glucan of at least 4 ng/m^3 (G-high) and 14 subject living in houses with levels of airborne glucan of less than 3 ng/m^3 (G-low), were exposed to grifolan suspended in saline as well as to saline alone. Comparing the groups before exposure showed that the G-high group had an increased TNFα secretion from the blood mononuclear cells and a lower number of cytotoxic T cells in blood compared to the G-low group. These results suggest that persons living in homes with higher levels of glucan (indicator for molds) show changes in the inflammatory and immunological system. The inhalation exposure to saline induced an increase in the secretion of TNFα, IFNγ and IL-10 in PHA-stimulated blood mononuclear cells, while the exposure to the combination of glucan and saline abrogated the saline effect. This down regulation of the saline induced inflammation by glucan is in accordance with previous results in animals exposed to endotoxin and glucan.

KEY WORDS:

(1→3)-β-D-glucan, inhalation, tumor necrosis factor α, cytotoxic T cell

INTRODUCTION

Numerous reports describe an association between molds and dampness in buildings and the presence of symptoms from the airways as well as general fatigue and headache. The character of the symptoms suggests that they be caused by a non-specific inflammation rather than by an allergic reaction [Rylander et al. 1992]. There are various species of fungi containing (1→3)-β-D-glucan in the cell wall. It consists of a polyglucose with a β (1→3) linkage and appears in a variety of forms with single or triple helix molecular structures, unbranched or branched to different degrees [Yadomae and Ohno 1996]. (1→3)-β-D-glucan have a variety of effects on immune competent cells [Di Luzio 1985, Williams 1997].

Inhalation studies of glucans in animals have revealed an alteration of the inflammatory response to endotoxin with following consequences for the accumulation and activation of cells in the lung tissue and airways [Fogelmark et al. 1994, Rylander and Holt 1998, Fogelmark et al. 1997]. Previous studies have demonstrated a relation between the level of (1→3)-β-D-glucan indoors and the extent of different kinds of symptoms [Rylander et al. 1996, 1998]. Investigations in a rowhouse area demonstrated a relation between the blood level of myeloperoxidase and the level of (1→3)-β-D-glucan in the homes. A decrease in forced vital

capacity during one second (FEV_1) and an increased prevalence of atopy among those living in houses with higher levels of (1→3)-β-D-glucan was also found [Thorn and Rylander 1998].

To further elucidate the role of (1→3)-β-D-glucan in the development of the inflammatory response seen among persons in moldy buildings, inhalation challenges with the pure substance were undertaken. The study comprised persons living in houses with higher levels of (1→3)-β-D-glucan and those living in houses with low or non-detectable levels. The two groups were compared regarding baseline values and the reactions seen after inhalation of pure (1→3)-β-D-glucan. The following is a preliminary report from the ongoing study.

METHODS

Persons

The persons were recruited from the rowhouse study mentioned above as well as by advertising. Based on the measurements of (1→3)-β-D-glucan in the homes, two groups were identified - 14 persons with an average level lower than 3 ng/m³ (G-low) and 14 persons with an average level above 4 ng/m³ (G-high). Persons between 19 and 65 years old, without asthma, cardiovascular diseases or other disease that could put the person at risk during the experiment, were invited to participate. Table I shows the number of persons and the personal characteristics.

TABLE I: The results from measuring (1→3)-β-D-glucan at home (ng/m³) and basic characteristics of the subjects. G-low = subjects in homes with low and G-high = subjects in homes with high levels of glucan. Standard deviation in parenthesis.

	G-low	**G-high**
n	**14**	**14**
Glucan in the home		
- mean	1.19 (0.86)	6.07 (2.18)
- range	0.18 - 2.70	4.2 - 11.0
Females	9	6
Atopic	7	3
Smokers	0	2
Age		
- mean	55	41
- range	35 - 66	19 - 61

Exposure

One to three persons were exposed at the same time and each subject was exposed to (1→3)-β-D-glucan in saline and to saline alone, as a control, on two randomized occasions. For the glucan exposures, grifolan was used, a water-nonsoluble (1→3)-β-D-glucan extracted from *Grifolanum commune* (generous gift from prof N Ohno, Tokyo, Japan). The exposures were performed in a chamber, 2x3.5x2.45 m, furnished as a sitting room. The solution was aerosolized and led into the exposure chamber through the ventilation system. Sampling of the air in the room was done with Isopore filters. The exposure time was three hours.

The amounts of airborne (1→3)-β-D-glucan on the filters were determined using a specific Limulus lysates (Fungitec G Test®, Seikagaku Co, Tokyo, Japan) [Tamura et al. 1994]. The filters were also analyzed for endotoxin using specific endotoxin lysate (Endospecy®). No endotoxin was detected on the filters. The average concentration of (1→3)-β-D-glucan in the chamber was 30 ng/m^3 (range 15.3 - 43.2 ng/m^3) which was about 3-30 times higher than the glucan concentration measured in the persons homes. The total dose of (1→3)-β-D-glucan inhaled during the three hours exposure was approximately 20 ng.

Total and differential blood cell count and lymphocyte subsets

Blood was collected in EDTA tubes and total leukocyte count was made in light microscope. Differential cell counts were made on smears stained with May-Grünwald-Giemsa and counted in light microscope.

Lymphocyte subsets were quantified before exposure only, using flow cytometry. Helper/inducer (CD4+), cytotoxic/suppressor (CD8+) T cell populations were identified. CD8+ cells were also analyzed together with a monoclonal antibody binding S6F1, an epitope of the α-chain in the LFA-1 complex (CD11a). CD8+ T cells expressing S6F1 have been classified as cytotoxic cells, while CD8+ T cells with a week staining for S6F1 are either naive cells or suppressor effector cells [Morimoto et al. 1987].

Inflammatory markers and atopy

Eosinophilic cationic protein (ECP) was assayed in serum by a fluorescent enzyme immunoassay technique (CAP ECP FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden) and expressed as μg/l. Myeloperoxidase (MPO) was assayed by a radio immunoassay technique (CAP MPO RIA, Pharmacia Diagnostics AB, Uppsala, Sweden) and expressed as μg/l.

Atopy was determined by the concentration of specific serum IgE antibodies against ten airborne allergens using a fluorescent enzyme immunoassay technique (CAP Phadiatop FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden). The results were expressed as positive (atopic) or negative (non-atopic).

Cytokine production

Venus blood was collected before and 24 hours after exposure for cytokin analysis. Mononuclear cells (MNC), 1x10^6/ml, were suspended in AIM-V medium (Gibco BRL) supplemented with 2-mercaptorthanol, 4x10^{-5}M, and incubated with or without phytohaemagglutinin (PHA, Murex Diagnostics Limited), final concentration 500 ng/ml or LPS, 500 pg/ml. After 48 hours incubation at 37ªC, 5% CO2, the supernatants were collected and stored at -25ªC till cytokine analysis. TNFα from unstimulated MNC was analyzed using an ELISA kit with a sensitivity of 0.1 pg/ml (Quantikine high sensitive, R&D Systems). IL-10, IFNγ and TNFα from PHA stimulated MNC were analyzed using PeliKine-compact™ human cytokine ELISA, CLB, The Netherlands.

RESULTS

COMPARING THE GROUPS BEFORE EXPOSURE

Table II shows the results from the lymphocyte subpopulation quantification. The number of cytotoxic T cells, CD8+S6F1+, was significantly lower in the G-high group compared to the G-low group, also reflected in the higher ratio for CD4+ and CD8+ lymphocytes in the G-high group.

TABLE II: Number of different subclasses of T lymphocytes (10^9/l) among persons with a low level of glucan in their homes (G-low) and persons with a high level (G-high). Mean and standard deviation in parenthesis. * = $p<0.05$ comparing G-high to G-low.

	G-low	**G-high**
n	**14**	**13**
CD4+	0.79 (0.24)	0.82(0.29)
CD8+	0.48 (0.13)	0.43 (0.36)
CD4+/CD8+	1.65 (0.66)	2.53 (1.33)
CD8+S6F1+	0.34 (0.17)	0.27 (0.32)*
CD8+S6F1-	0.24 (0.17)	0.24 (0.11)

Table III shows the values of TNFα secretion in non-stimulated cells. Due to technical faults, some analyses had to be discarded and only nine persons in the G-high group were analyzed for cytokines. The G-high group had a significantly higher baseline secretion of TNFα in non-stimulated MNC compared to the G-low group ($p=0.04$). The secretion of IL-10 and IFNγ in non-stimulated MNC were below the detection limit in most cases. In PHA-stimulated MNC there were no significant differences between the two groups.

TABLE III: Secretion of TNFα from blood mononuclear cells (pg/ml medium) among persons with a low level of glucan in their homes (G-low) and persons with a high level (G-high). Mean values and standard deviation in parenthesis. * = $p<0.05$ comparing G-high to G-low.

	G-low	**G-high**
n	**14**	**9**
TNFα		
Unstimulated	7.1 (3.5)	11.3 (6.9)*
PHA-stimulated	1510 (2940)	1564 (2722)
LPS-stimulated	98 (84)	63 (40)

RESULTS AFTER EXPOSURE

No effects were found in MPO, ECP, total leukocyte count, monocytes, lymphocytes or neutrophils in blood. Table IV shows the results for blood eosinophils. The G-high group had a larger increase in eosinophils immediately after both exposures, significantly after saline exposure, compared to the G-low group.

Exposure to saline alone induced an increase in secretion of TNFα, IL-10 and IFNγ in stimulated MNC. The exposure to saline+glucan abrogated this increase, so that the majority of the

persons had a lower cytokine secretion after the exposure compared to before. This reaction was most pronounced in the G-low group. Table V shows the results for TNFα.

TABLE IV: Eosinophils in blood (10^9/l) in subjects living in homes with low (G-low) and high (G-high) levels of (1→3)-β-D-glucan in their homes. Mean and standard deviation in parenthesis.
*= $p<0.05$ comparing G-high after exposure value to baseline.

	G-low	G-high
n	**14**	**14**
Baseline	0.15 (0.10)	0.18 (0.11)
After NaCl	0.15 (0.10)	0.26 (0.19)*
Baseline	0.15 (0.09)	0.17 (0.11)
After grifolan	0.16 (0.09)	0.20 (0.13)

TABLE V: Changes in cytokine secretion from blood mononuclear cells (pg/ml medium) induced by exposure to saline and to glucan suspended in saline (S+glucan). G-low = subjects in homes with low and G-high = subjects in homes with high levels of glucan. Standard deviation in parenthesis.
** = $p<0.01$ comparing saline exposure to saline+glucan exposure.

	Saline	S+Glucan
G-low, n=14		
Unstim	-0.12 (3.75)	-0.59 (3.53)
PHA stim	379 (812)	-400 (732)**
G-high, n=9		
Unstim	-0.83 (4.92)	-0.04 (3.53)
PHA stim	478 (847)	55.2 (497)

DISCUSSION

The results are so far based upon a small number of persons and should thus be interpreted with caution. The data show that the major effect related to living in a house with higher levels of (1→3)-β-D-glucan, was a decreased number of cytotoxic T lymphocytes in blood and an increased baseline secretion of TNFα from MNC. These data could be the result of an ongoing low-grade chronic inflammation in the G-high group, characterized by an enhanced trapping of cytotoxic CD8+ T cells in the lung tissue or airway epithelium. The S6F1 is a marker of cytotoxic effector cells and it has been shown that only CD8+ T cells expressing S6F1 migrate through endothelium [Berman et al. 1995]. The only study previously published on mold exposure and lymphocytes was carried out in children. It showed changes in the numbers of subsets of T cells comparing children in high and low fungi contaminated homes. Contrary to our results, the data showed a lower CD4+/CD8+ ratio among mold-exposed children [Dales et al. 1998].

Exposure to saline alone induced an increase of the *in vitro* PHA stimulated cytokine secretion from MNC. However, the combined exposure to saline and glucan abrogated the saline induced cytokine secretion. These results are in accordance with previous *in vitro* experiments, showing that (1→3)-β-D-glucan is phagocytosed by alveolar macrophages and that the endotoxin induced TNFα secretion is abrogated in glucan pretreated macrophages [Goto et al.

1994]. In guinea pigs, exposure to endotoxin induces a migration of neutrophils into the airways, while preexposure to (1→3)-β-D-glucan decrease this neutrophil influx [Fogelmark et al. 1994, 1997]. In summary, the results indicate that acute exposure to glucan involves down regulating mechanisms. The results showing increasing eosinophil numbers in blood after exposure in the G-high group, might be an effect from long time exposure to glucan. Preliminary data from animal studies show increased numbers of eosinophils in the airway epithelium after a five weeks exposure to (1→3)-β-D-glucan.

CONCLUSION

The results suggest a relation between living in homes with higher levels of glucan (indicator for molds) and changes in the inflammatory and immunological system. The increased TNFα secretion from blood cells indicates an ongoing inflammation. An increased trapping of CD8+ T cells in the airways could cause a lower number of these cells in blood. The glucan induced down regulation of the inflammatory reaction seen after saline exposure is in accordance with previous results in animals exposed to endotoxin and glucan.

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BUILDING-RELATED ILLNESS IN OCCUPANTS OF MOLD-CONTAMINATED HOUSES: A CASE SERIES

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ABSTRACT

The clinical presentation and course of individuals and families who developed illnesses related to non-infectious fungal exposures inside their homes is described. Occupants developed their illnesses shortly after their homes had been water damaged. A few occupants had a specific building-related illness, such as hypersensitivity pneumonitis or asthma exacerbation, but most had a "sick building syndrome" symptom complex involving irritation/inflammation of the mucous membranes, respiratory tract, and skin; fatigue; and/or neurocognitive dysfunction. All cases required months or years to correctly diagnose. Air, surface, and/or bulk microbiological sampling in most of the homes yielded high concentrations of toxigenic fungi, including *Stachybotrys chartarum* and *Penicillium* and *Aspergillus* species, emanating from water-damaged building materials. Most of the ill individuals had complete clinical improvement shortly after their removal from the contaminated indoor environment, but a few individuals continued to experience symptoms in response to a variety of environmental irritants. The author proposes a new clinical syndrome entity to describe the non-infectious, mold-related, building-related illness.

KEY WORDS:

Building-related illness, sick building syndrome, toxigenic fungi, environmental disease, *Stachybotrys chartarum*, hypersensitivity pneumonitis.

INTRODUCTION

Building-related illnesses (BRIs) have been recognized for many years as occupational or environmental diseases. Specific disease entities such as asthma, hypersensitivity pneumonitis (HP) (Banaszak et al. 1970; Bernstein et al. 1983), and legionellosis have been described in relation to a number of chemical, allergenic and infectious agents. The "sick building syndrome" (SBS), in contrast, remains a clinical and epidemiological entity for which a specific toxicological, microbiological, allergic or other etiology has not been determined clinically or environmentally (Hodgson 1995). In SBS, a significant percentage (undefined) of building occupants complains of a combination of respiratory, mucous membrane, constitutional, skin, and/or neurocognitive symptoms that occur predictably when the occupants are inside the building environment and which improve when they are away from the building. "Multifactorial" etiologies, including inadequate ventilation (Stenberg et al. 1994), off-gassed organic vapors from building materials, and psychogenic factors (Skov et al. 1989), have been implicated in published

cross-sectional and retrospective epidemiological studies of such "sick" buildings, but to date no unifying etiology has been demonstrated. Air quality investigations of buildings where occupants complain of these unexplained, building-related symptoms have typically focused on sampling for chemical (e.g., carbon monoxide, formaldehyde, fiberglass), allergic, and infectious agents (Menzies et al. 1996; Hodgson 1995). In most such cases, no abnormal contaminant is found and the building is labeled as "sick," suggesting an idiopathic etiology. Cases and studies of "sick" buildings published in the medical literature have mostly involved commercial office buildings and manufacturing facilities (Burge et al. 1987), with fewer reported cases of residential dwellings (Croft et al. 1986).

The role of water damage to and fungal colonization of building materials, with detection of fungi in air and/or on surfaces at high concentrations, is increasingly being recognized as a specific cause of some BRIs (notably asthma and HP), and possibly the sick building syndrome itself (Hodgson et al. 1998; Johanning et al. 1996; Sudakin 1998). In some "sick" buildings, external or internal water damage may not be recognized or detected until after an initial air quality investigation for non-microbiological causes has been completed. Several recently published clinical- epidemiological studies of buildings with occupants with SBS symptoms have identified the presence of high airborne or surface concentrations of fungal spores, trichothecene mycotoxins, and microbiological volatile organic chemicals (MVOC) produced by certain fungi which are associated with clinical symptomatology (Johanning et al. 1996). However, large gaps in clinical, pathological, epidemiological, and exposure assessment remain in determining the causal relationship between indoor fungal contamination and the SBS (Smoragiewicz et al. 1993).

MATERIALS AND METHODS

Five (5) cases evaluated in private occupational/environmental medicine (OEM) consultation practice by the author are presented. The cases are comprised of individuals or families who were either self-referred (n=2), referred by an industrial hygienist (n=2), or referred for evaluation by a workers' compensation insurer (n=1) for consultation. The history of illness, its relationship to the home environment, the medical evaluation(s) and treatment(s), and past medical, occupational and environmental history were obtained for each case. The history of building-related water damage, if known, was reviewed. Walk-through inspection of the homes was performed by the author in 4 of 5 cases. Environmental microbiological testing of surface, bulk, and air samples, either before or after the clinical evaluation, was obtained in all cases. Occupants' clinical response to removal from the mold-contaminated home environment were documented.

RESULTS

Results of the five cases are summarized in the Tables (Cases #1-5) below. All of the cases occurred in Nevada or Utah. Details of the clinical and microbiological investigations for each case are available upon request from the author.

TABLE I: Mold-Contaminated Houses - A Case Series.

Case	1
Subject(s)	78 year old, single woman
Description of Home	50 year old, 3-story house
Structural Damage to Home	Plumbing fixture burst on top floor, flooding walls, ceilings, carpets, floors. Two inches of standing water has been in basement. Partial remediation occurred without containment. Not all wet materials were removed; others were painted over or sprayed with fungicide.
Clinical Illness	Subject had a history of stable bronchiolitis obliterans with mild, chronic cough. After entering the partially-remediated house, she developed immediate symptoms of fatigue, weakness, sore throat, chest pressure, and nasal congestion, followed by delayed-onset, flu-like illness (fevers, chills, cough, dyspnea) that worsened after several hours in the house.. Re-entry challenge reproduced fever, chills, dyspnea and chest pressure symptoms, with new infiltrates on chest x-ray and hypoxemia.
Duration of Symptoms before Diagnosis	6 months
Microbiological Sampling Results	Surface wipe sampling of dust throughout the house demonstrated a variety of atypical culturable fungi of various species, notably *Penicillium auranteogriseum*, with maximum concentration of $1.7x10^6$ CFU/in^2 (75% predominant) in basement.
Diagnosis	HP; "SBS-like illness"
Intervention and Outcome	Removed from home, upon which symptoms resolved. Only baseline cough persisted.
Comments	Homeowners' insurance company insisted home was habitable. Subject initiated a lawsuit against homeowners' insurance company and remediation contractor.
Case	2
Subject(s)	40 year old, male, employed as a heavy equipment mechanic, and his family (wife 38, two teenage children)
Description of Home	Rental, one-story townhouse, 10 years old. Family was not aware of any history of water damage to the house.
Structural Damage to Home	Subject and family noted only black discoloration underneath linoleum in the bathroom upon moving into townhouse; they did not realize there was water damage. Bathroom and adjacent walls were extensively water damaged. Subsequently extensive water damage to subfloor and standing water in crawl space was detected.The cause of water damage was chronic plumbing leaks and surface infiltration of underground aquifer.
Clinical Illness	Subject complained of persistent sinus congestion, rhinitis, sore throat, swollen cervical lymph nodes, cough productive of brown and green sputum, fevers with night sweats, and profound, unex-

Table I cont.

	plained fatigue. Dyspnea, cough, and night sweats became predominant. He had one chest x-ray at the time he was symptomatic which was interpreted as "pneumonia," with pO2 74. He was misdiagnosed with bacterial endocarditis and treated with IV antibiotics for 8 weeks. He improved during hospitalization but symptoms recurred when he returned home. Subject and wife, both smokers, had abnormally elevated carboxyhemoglobin (COHb) levels out of proportion to smoking; house carbon monoxide levels were undetectable. Wife and children had similar but less severe symptoms, without fevers or sweats. Pet cat's behavior became bizarre.
Duration of Symptoms before Diagnosis	2 years
Microbiological Sampling Results	Bulk sample of bathroom moulding: *Aspergillus versicolor* ($7.0x10^6$ CFU/gm), *Chaetomium* ($3.4x10^6$ CFU/gm), and *Penicillium* fungi, with low concentrations of thermophilic bacilli and *Thermoactinomyces candidus*. Carpet, pad, wall sheetrock and flooring tack strips in water-infiltrated areas of the hallway adjacent to the bathroom: extremely high concentrations (10^6-10^8 CFU/gm) of *Aspergillus, Penicillium, Fusarium*; plus spores of *Stachybotrys chartarum* and thermophilic actinomycetes.
Diagnosis	(?)HP; "SBS-like illness"
Intervention and Outcome	Family was removed from home. All occupants' principal symptoms resolved within 2 weeks.
Comments	Subject screening precipitants (IgG) panel was negative. (N.B. fungal antigens did not match those found in the house). Re-entry challenge test was recommended to establish the diagnosis of HP but landlord prevented further testing from being conducted.
Case	3
Subject(s)	Family: Mother (38), Father (38), 3 children (10, 12, 13)
Description of Home	New, pre-fabricated, modular, 1-story house with basement
Structural Damage to Home	Water heater exploded in basement. Basement wall, ceiling, and carpet were soaked. Family tried to remove water with fans, left wet materials intact.
Clinical Illness	Within one week of flooding, family members developed recurrent "head colds" and "flu." Children developed papular rash over arms and legs and school performance declined. Son with history of mild asthma developed significant worsening of his disease. Mother developed marked behavioral changes, difficulty concentrating and short-term memory impairment, profound fatigue, and persistent facial edema.
Duration of Symptoms before Diagnosis	1 ¼ years

Table I cont.

Microbiological Sampling Results	Air sampling for culturable fungi: >4.6x10^4 CFU/gm of *Penicillium, Aspergillus, Cladosporium*, and other fungi. Bulk samples of carpet and pad: 10^4-10^5 CFU/gm *Aureobaidium, Penicillium*, and other fungi. *Stachybotrys chartarum* spores and conidiophores were identified on virtually all wet sheetrock samples, and in air samples. (Burkard personal impactor).
Diagnosis	"SBS-like illness;" asthma exacerbation (son); MCS (mother)
Intervention and Outcome	Family was removed from home. All improved except mother who had persistent neurocognitive impairment.
Comments	Homeowners' insurance offered only to "patch" water-damaged areas; felt house was habitable. House was foreclosed upon by mortgage holder for failure to occupy premises. Family initiated a lawsuit against their homeowners' insurance company.
Case	4
Subject(s)	Wife (43) and husband
Description of Home	Brand new, double-wide trailer transported from show lot in another state. Modular components were reassembled on-site before occupancy.
Structural Damage to Home	Unknown. Author speculates that during transport of two "halves" of house, ceiling insulation (shredded newspaper) and wall insulation (fiberglass) were not properly covered and received water infiltration from rain.
Clinical Illness	Within 2 weeks of moving into house, wife developed bizarre personality and mood changes, including becoming verbally abusive to her husband; metallic taste in her mouth; eye irritation; dryness of the nasal mucosa; insomnia; difficulty speaking; fatigue; and difficulty concentrating on simple tasks. Any irritant chemical triggered her symptoms. Both wife and husband developed papular rashes over their bodies. Husband was otherwise asymptomatic.
Duration of Symptoms before Diagnosis	6 months
Microbiological Sampling Results	Burkard air and bulk samples yield *Aspergillus, Penicillium*, and yeasts in furnace filter, attic insulation and wall insulation after destructive testing 2 years after house was first occupied. House had a yeast-like odor.
Diagnosis	"SBS-like illness;" MCS
Intervention and Outcome	Family vacated house. Husband improved; many of wife's symptoms resolved but chemical "sensitivity" remained, as well as persistent fatigue.
Comments	Homeowners' insurance refused to acknowledge building-related problem, felt house was habitable. House was foreclosed upon by

Table I cont.

	mortgage holder. Family initiated a lawsuit against their homeowners' insurance company to remediate house.
Case	**5**
Subject(s)	38 year old, singe female
Description of Home	One-story, 2-bedroom cottage in high-end residential neighborhood.
Structural Damage to Home	Unknown for 1 ½ years. Discovered "mushrooms" and "black, tar-like substance" above dishwasher in cabinet and on wall. Dishwasher discharge hose had been leaking onto the concrete slab, with water migrating up the wall.
Clinical Illness	Approximately one month after moving in, she developed a variety of unexplained symptoms, including extreme fatigue, axillary pain, maculopapular rash, choking sensation, and hoarseness. Exposure to common chemicals triggered severe symptoms.
Duration of Symptoms before Diagnosis	2 years
Microbiological Sampling Results	High concentrations (>4.6x10^4) of *Stachybotrys chartarum* spores were measured in air (Burkard) samples, while culturable (Andersen) air samples yielded predominantly *Aspergillus, Penicillium, Chaetomium*, and other fungi. Scotch tape and surface sampling throughout the house demonstrated these fungal species, including *Stachybotrys* spores on the kitchen ceiling fan blade and in the portable air filter in the living room.
Diagnosis	"SBS-like illness;" MCS
Intervention and Outcome	Subject moved out of house. Most of her symptoms resolved but chemical "sensitivity" remained.
Comments	Homeowners' insurance refused to acknowledge building-related problem, felt house was habitable. House was foreclosed upon by mortgage holder. She initiated a lawsuit against her homeowners' insurance company.

Abbreviations: HP= Hypersensitivity pneumonitis. SBS= Sick building syndrome. MCS= Multiple chemical sensitivity syndrome.

DISCUSSION

These five cases illustrate important clinical features and issues in the clinical approach to and diagnosis of disorders related to indoor environmental fungal (mold) contamination in the home environment. In all of the cases described, individuals (occupants, or subjects) developed their illness within a few days to weeks of living in a mold-contaminated environment, though in most of the cases the individuals were unaware of the presence or significance of the water damage and/or mold contamination. Moldy odors were not appreciated by all exposed indi-

viduals. All subjects incorrectly attributed their illness to non-environmental causes for many months while continuing to reside in their contaminated houses.

Some individuals developed what was clinically suspected to be a specific BRI, namely HP (Case #1 and 2) or exacerbation of asthma (Case #3). The HP cases were clinical diagnoses which could not be confirmed through antibody testing or tissue biopsy, primarily because of financial and legal constraints. One of two HP cases was clinically confirmed through an actual exposure challenge test in the home environment, with physician-observed evidence of delayed-onset fevers, chills, and dyspnea; bilateral lower-lobe pneumonitis on chest x-ray (superimposed upon a pre-existing, chronic lung disorder that was presumed to be bronchiolitis obliterans); and hypoxemia documented by arterial blood gas. In both of these suspected HP cases, there were other clinical features, such as upper respiratory tract inflammation (sore throat, nasal congestion) and fatigue which were not delayed in onset with exposure to the building environment, and which appeared to overlap with the HP (or HP-like) symptoms.

In contrast to the limited number of BRIs described above, most individuals experienced a "SBS-like" illness defined by irritation of mucous membranes of the eye, nose, and throat; upper and possibly lower respiratory tract irritation, marked by cough, chest tightness, and dyspnea; profound, unexplained fatigue and related constitutional symptoms; evanescent, scattered rashes over the face, neck, torso, and extremities; and/or neurocognitive dysfunction, manifested by difficulty concentrating, short-term memory impairment, mood irritability, and other behavioral disturbances. The distribution and severity of symptoms varied among cases, even within households. Both children and adults were affected.

The diagnosis of an environmental etiology of the fungal contamination became evident in all but one case (#4). Internal and external water damage to each of the home from a variety of causes produced microbiological colonization of building materials in various locations in the occupied space and building envelope. The etiology for illness in Case #4 was never confirmed. It was speculated that the attic insulation (made of shredded newspaper) had not been properly covered during transport and had become wet, such that when the house was sealed, the moist, dirty, cellulose insulation became a source of colonization of fungi. The potential route of exposure to the occupied space was hypothesized to occur through the gap joining the two halves of the "double-wide" house when the (joined) interior space became negatively pressurized as doors in the house were opened and closed.

All of the cases were notable for a significant delay (range: 6 months to 2 or more years) between the onset of symptoms and the time to a definitive diagnosis of environmental disease, including referral to an OEM physician and/or a certified industrial hygienist (CIH). Environmental histories were notably absent from occupants' medical evaluations by their family physicians, internists, and medical specialists. In one case (#2), the (mis)diagnosis of endocarditis (which was suspected because of unexplained fevers and "confirmed" by 1 out of 4 blood cultures positive for what was most likely a skin contaminant during the blood draw) seriously delayed the diagnosis. The subject's (#2) clinical improvement during his hospitalization was deemed to confirm his diagnosis of endocarditis, when in fact he improved clinically because he had been removed from his home environment. Failure to obtain an adequate, or for that matter any occupational and environmental history continues to be problematic for physicians in primary care and medical specialties (McCurdy et al. 1998). In buildings with rel-

atively few occupants, such as these residential cases, illness among a small number of occupants may not raise occupants' suspicion of an environmental etiology. The delay in recognition of an environmental etiology by the occupants themselves may have been due in part to the fact that they spent so much time in their homes that they did not notice an environmental relationship to their symptoms. Individuals who present to physicians with a symptoms-complex of persistent or recurrent rhinitis, sore throat, cough, chest tightness, and fatigue are often (mis)diagnosed as having (viral or bacterial) upper respiratory tract infections, asthma, or "allergies," and are often (inappropriately) treated with oral antibiotics. Alternative (differential) diagnoses or etiologies, even after multiple visits for the same complaints, are typically not considered. Neurocognitive symptoms, as described above, differentiate the mold-related environmental syndrome from these common infectious respiratory tract ailments, but are often not elicited by physicians or volunteered by patients in the presence of the other prominent, "organic" symptoms which brings these patients to seek medical attention.

Most occupants in this residential case series developed symptoms consistent with those described in cases of "sick building syndrome," namely a constellation of mucous membrane (eye, nose, throat) irritation; upper and possibly lower respiratory tract inflammation or irritation (rhinitis, nasal congestion, hoarseness, cough, dyspnea, chest pain); prominent fatigue, headache, and malaise (constitutional); sometimes skin lesions (erythematous, papular rash); and neurocognitive dysfunction (difficulty concentrating, behavioral changes, fatigue, headache), which occur upon or shortly after entering a particular building, and which improve after leaving the building. Physical examination and laboratory test results were unrevealing late in the course of nearly all cases (except #1), particularly with regard to objective evidence of lower respiratory tract disease. Rashes, nasal mucosal inflammation, and wheezing in (pre-existing) asthmatics were the most consistently documented examination findings.

Currently available immunological tests such as fungal IgE and IgG antibodies, have been shown to be of low sensitivity and specificity as markers of exposure or disease in non-infectious, mold-related illness in the absence of a specific (i.e., Type I-,III-, or IV- immunologically-mediated) BRI such as asthma or HP (Hodgson et al. 1998; Johanning et al. 1996; Malkin et al. 1998; Rylander 1994). Even when the clinical presentation is consistent with HP, the diagnosis of HP is not always able to be confirmed immunologically, particularly when more than one potentially immunogenic fungal (or bacterial) antigen is detected environmentally. The finding of unexplained, elevated carboxyhemoglobin levels in one case (#2) warrants further exploration of its clinical significance and potential as a marker for fungal bioaerosol exposure.

The role of certain fungal genera and species, particularly toxigenic fungi which thrive indoors in areas of high water activity such as on water-saturated gypsum board and ceiling tiles, is becoming increasingly implicated as the cause of certain building-related illness that is clinically indistinguishable from sick building syndrome (Hodgson et al. 1998; Johanning 1996; Sudakin 1998). Current theories about the pathogenesis of this mold-related illness suggest that symptoms may be caused by a combination of an allergic-type response to fungal components, and/or systemic toxicity or neurotoxicity due to mycotoxins and/or microbiological volatile organic chemicals (MVOCs) through several potential mechanisms (Sorenson et al. 1987). The clinical illness does not appear to represent an infectious pulmonary or systemic fungal disease, nor does it appear to be mediated through a classic, immediate (IgE-mediated)

allergic mechanism. Symptoms are not always consistent among similarly exposed individuals, and some exposed individuals' illness often become more severe over time, thus impeding an estimation of whether a exposure-response relationship exists. Furthermore, occupants' subjective complaints often are not confirmed by objective evidence of organ dysfunction, particularly in the case of possible obstructive lower respiratory tract disease (Muzi et al. 1998). Limitations in sampling, quantification, and speciation of indoor fungal contaminants also confound exposure assessment and estimation of dose-response and immune-response relationships.

These limitations in the understanding the pathophysiology of disease, and inconsistent approaches to case definitions in epidemiological investigations, have impeded the general acceptance and definition of a non-infectious, mold-related disease entity within the medical and scientific communities (Smoragiewicz et al. 1993). The author therefore proposes the term "NIFIES" (pronounced "knife-eez," for Non-Infectious Fungal Indoor Exposure Syndrome) to designate and recognize this specific BRI caused by indoor mold exposure in residential and commercial buildings. As clinical, epidemiological, microbiological, and toxicological research elucidates a specific disease mechanism, this syndrome may be replaced by a more specific disease appellation, and may replace the term "SBS" as well.

Three of five cases in this report had *Stachybotrys chartarum* identified as an indoor contaminant. The presence of *Stachybotrys chartarum*, which has received considerable attention from one case associated with dramatic health effects unrelated to SBS-like symptoms (Montana et al. 1997), has been observed in numerous occupational and residential cases where one or more species of toxigenic fungi have been environmentally detected in high concentrations on building material surfaces and/or in air (Sorenson et al. 1987). The clinical significance and human toxicology and immunology of *Stachybotrys chartarum* and other fungal trichothecene mycotoxins remains incompletely understood at present (Fung et al. 1998). The clinical illnesses in these cases where *Stachybotrys* was identified can therefore not be solely attributed to this particular organism in the presence of other genera of toxigenic fungi such as *Penicillium*, *Aspergillus*, and *Chaetomium*.

Most of the occupants who were diagnosed with a BRI or "SBS-like illness" (or, as suggested above, NIFIES) had marked clinical improvement after removal from the residential environment. Remediation of the water-damaged and mold-contaminated house was attempted in only one (#1) case, with paradoxically greater indoor contamination created by lack of appropriate containment during remediation of mold-contaminated building materials. However, even after occupant removal, in three cases (#3,4,5) some occupants continued to experience primarily neurocognitive symptoms, though most of their other respiratory, skin, and mucous membrane symptoms had subsided. Two of these individuals, both female, experienced recurrent, predictable episodes of triggering of neurocognitive and other symptoms upon exposure to a variety of environmental chemicals and substances. This clinical scenario is consistent with the clinical entity of multiple chemical sensitivity (MCS). Like SBS, MCS remains a controversial disorder whose etiology remains poorly understood (Simon et al. 1993). This clinical observation nevertheless suggests that MCS may be a final common pathway for environmental insults that are not classically due to "toxic" chemical exposures. This finding of "bioaerosol-induced" MCS, albeit unexplained, may represent another possible long-term complication

from exposure to airborne concentrations of certain indoor fungi. Many of the scientific problems and issues surrounding MCS research and clinical validation are applicable to these cases, since they can only be studied retrospectively and lack any baseline neurobehavioral data for comparison (Fiedler et al. 1997).

Finally, from a practical and clinical standpoint, all of the cases were notable for conflicts caused by homeowners' or other insurers delay in or refusal to acknowledge the health hazards of indoor fungal contamination, the authorization of timely and qualified medical evaluation of occupants, and the provision of insurance coverage and relief for these homeowners or occupants. Legal intervention ensued in all of the cases. Two cases (#2 and 5) involved occupants who were not aware of water damage which had pre-existed their occupancy of the home. In one case (#2) which involved controversy over the issue of whether the medical condition was work-related, the workers' compensation process required lengthy legal appeals to substantiate a complete OEM investigation by the author, further delaying the investigation of the subject's residential environment and ultimately the correct diagnosis for many months. Once the environmental etiology in Case #2 was finally established, the property owner evicted the tenant and then demolished the contaminated portion of the house (evidence) before a diagnosis of HP could be confirmed. Reasons proffered by homeowners' insurance representatives (including lawyers and retained medical "experts") included refusal to recognize non-infectious, mold-related illness as a "valid" medical condition, and lack of "objective evidence" such as toxicological tests, allergy tests, or lung function tests to "prove" that the exposure occurred and that it caused the clinical illness. Insistence by insurers for industrial hygienists to "test for chemicals" (e.g., Case #4) when no such chemical exposures are evident from history and building inspection also delayed appropriate microbiological investigation. Finally, lack of familiarity with the role of a qualified OEM physician in conducting a building investigation appeared to be common factors in some insurers' decision to delay or deny a clinical investigation. These problems led to significant emotional and financial strain on affected occupants, particularly those who were forced to abandon their homes and continue paying mortgages.

CONCLUSION

Exposure to fungal contamination of water damaged houses and other buildings causes an SBS-like symptom complex and, to much lesser extent, specific, recognizable building-related diseases. Diagnosis and treatment of mold-related indoor illness is often delayed due to problems with medical evaluation, insurance claim acceptance, and delays in obtaining qualified, focused investigation of the home environment. Removal from (or remediation of) the contaminated environment usually but not always results in clinical improvement of affected individuals. The term "non-infectious, fungal indoor exposure syndrome" (NIFIES) is proposed to define the illness described herein, as well as in other similar cases reported in mold-contaminated occupational and environmental settings, as a unique disease entity. Further clinical, epidemiological, microbiological, and other scientific research is needed to elucidate the mechanisms of disease, and to identify and quantify sources and routes of human exposure to fun-

gal bioaerosols. The determination of whether NIFIES is indeed the same clinical entity as sick building syndrome also awaits these advances.

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DIAGNOSING THE CAUSE OF A "SICK BUILDING:" A CASE STUDY OF AN EPIDEMIOLOGICAL AND MICROBIOLOGICAL INVESTIGATION

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ABSTRACT

This report describes the methods and outcome of a physician-led investigation of occupants' prolonged, unexplained illnesses associated with working inside a large, modern office building. Occupants (cases) complained of building-related symptoms including eye, nose, and throat mucous membrane irritation; rashes; respiratory symptoms; profound, unexplained fatigue; and neurocognitive symptoms, including difficulty concentrating and short-term memory impairment. No functional ventilation problems or chemical contamination were detected in a walk-through evaluation and basic air quality testing. An epidemiological survey of the building's 700 occupants was then conducted. With 86% of the occupants responding to the survey, there was an average case prevalence of health-related complaints of nearly 25%, evenly distributed among floors. Cases were geographically distributed in a pattern which coincided with the location of the ceiling-mounted variable air volume (VAV) boxes which distributed ventilated air to the occupied spaces. Re-inspection revealed previously undetected, focal water-staining of 40% of ceiling tiles located underneath the VAV boxes throughout the building. Active growth of *Stachybotrys chartarum (atra)* and other fungi was detected on many of the damaged tiles. All water-damaged tiles were replaced and VAV hot water valves were tightened. Occupants reported significant improvement of symptoms within weeks after these changes.

KEY WORDS:

Sick building syndrome, toxigenic fungi, indoor air quality, environmental disease, *Stachybotrys chartarum (atra).*

INTRODUCTION

"Sick building syndrome" (SBS) is defined as a clinical and epidemiological entity in which a significant percentage of occupants of a building develop unexplained symptoms involving several organ systems, including the respiratory tract, skin, and nervous system. The term "sick

building" implies an unknown, environmental etiology for which, after investigation, no specific chemical, physical, infectious, or allergic causative agent has been identified (Hodgson 1995). Current hypotheses of SBS etiology continue to implicate multifactorial causes, including inadequate ventilation, organic vapors, asphyxiant gases (e.g., carbon monoxide) and psychogenic factors (Menzies et al. 1996). However, in recent years, an increasing number of published investigations of sick buildings have identified water damage of ventilation systems or building materials, with consequent growth of fungal contaminants, as the source of illness that is clinically identical to SBS. These cases have been associated with a variety of internal and/or external sources of water infiltration into the building environment, including humid climates (Hodgson et al. 1998), leakage from the building exterior (Johanning et al. 1996; Sudakin 1998), and contamination of the air handling units or ducts of the building's ventilation system. Such water damage may be acute or chronic, and may not have been previously identified as a problem during an indoor air quality (IAQ) investigation. Identification and removal of the offending fungi from the environment via removal or remediation of water-damaged materials or conditions, and/or removal of occupants from the source of exposure, usually reversed occupants' disease/complaints in these reported cases. The hydrophilic fungus, *Stachybotrys chartarum (atra)*, has received particular attention in many of these cases.

The case study reported herein represents a case of a "sick building" for which an etiology was not discovered until an epidemiological study and subsequently a focused, microbiological investigation revealed a previously unrecognized source of water damage to building materials with localized growth of toxigenic fungi and a recognizable route of dissemination of fungal spores to occupied spaces. Shortly after the building's opening, occupants began to sporadically report general air quality and health complaints related to the building. Many noted unexplained fatigue and difficulty concentrating, and what they or their personal physicians labeled as "constant head colds" and "recurrent bronchitis," though many did not initially attribute this to the workplace. Symptoms varied slightly over two years without a consistent pattern, with some questionable symptom and air quality improvement reported during the winter months. Many complaints were reported in one wing of the second floor housing the largest agency in the building, where occupants worked in two large partitioned rooms. Initial IAQ investigations and clinical evaluations of some ill occupants revealed no apparent environmental problem or contaminant. The building was labeled a "sick building."

MATERIALS AND METHODS

Building Description

The building is a modern, 5 story, state government office building located in Las Vegas, Nevada. It was constructed in 1992-94 and opened in 1994 to house approximately 20 state agencies. The building has an atrium which extends from the first through the fifth floor on the south side of the building. The building is completely sealed and is ventilated by eight separate, roof-mounted, air handling units (AHUs) that house heating, ventilation, and air conditioning (HVAC) and evaporative cooling components. Each AHU provides (outdoor or mixed) air to a vertical zone on all five floors of the building. The air is distributed to approximately 250 variable air volume (VAV) boxes throughout the building, each containing a secondary

heating coil unit with hot water supplied by a separate, treated water system. Air is exhausted from the occupied spaces via ceiling-mounted return grilles into a common, above-ceiling plenum in each zone. Return air is then exhausted from the building from rooftop-mounted vents that are located more than 50 feet from the air intake units.

Typical operating parameters varied depending on the season, with indoor humidity in the 40-50% range (typical outdoor relative humidity, 10-25%) and temperature ranging from 70 to 74ªF, reflective of the dry, hot, Las Vegas desert environment. During summer months when outdoor temperatures exceed 85ªF, the evaporative cooling system was utilized with return air re-circulated and mixed with outdoor air in a 20:80 ratio. Following persistent occupant complaints, the system was operated with 100% fresh air. The VAV units were operational during daytime hours, with minimum air delivery/minimum air exchanges during evening hours when the offices were not occupied.

Epidemiology

An anonymous occupant survey was designed to measure the types, prevalence, and distribution of symptoms among the building's occupants. The survey was distributed to 650 of the 700 total full- and part-time occupants in the building (50 occupants were not present during the survey period). All surveys were compiled for analysis by the occupational and environmental medical physician (JC). Information regarding age, sex, office location, number of hours worked, smoking habits, and number of years employed in the building was queried with logistic (yes/no) and continuous variable (integer) responses requested. Respondents were asked "Are you experiencing health problems now or in the past related specifically to working in this building?" with the choice of a logistic response. Subjects who answered "yes" to this question ("positive responders") were asked to answer a series of logistic and multiple-choice questions regarding pre-existing medical conditions (e.g., allergic rhinitis and conjunctivitis, asthma, chronic bronchitis, recurrent or chronic sinusitis, frequent headaches or chronic fatigue); characteristics of their building-related symptoms (e.g., timing, improvement away from the building, location, duration, lost work time); general work conditions (e.g., noise, temperature, lighting); and symptoms "specifically associated with working in the building" (e.g., upper respiratory; lower respiratory; skin; gastrointestinal; ocular; constitutional [headache, myalgia, fever/chills, exhaustion]; and neurocognitive [e.g., problems concentrating, memory problems, dizziness, fatigue, irritability, daytime drowsiness]). Demographic data (distribution of sex, ages, smoking habits) and symptom characteristics data were tabulated but were not statistically analyzed.

Positive responders who indicated at least one symptom in three categories other than gastrointestinal were counted as a "confirmed case." A "possible case" was defined as a positive responder who met the same criteria but who also indicated a pre-existing allergic, respiratory, or other medical condition that was aggravated by the building environment. All negative responders (i.e., those who answered no to building-related symptom question, or left this question blank and did not circle any symptoms) were classified as "non-cases." Seven surveys were rejected because of inconsistent responses. Subjective determination as to case category was made for surveys where the building-related symptom question was not completed but appropriate specific symptom responses were made. Responses were tabulated by general

office area and summary statistics were tabulated by office and floor. The office or desk location of all cases was tabulated and marked on a schematic layout for each floor in the building.

Environmental Exposure Evaluation

The methodologies for surface sampling using tape impression and swabs, and for air sampling using the Andersen single-stage impactor sampler (Graseby Andersen, Atlanta, GA) and the Burkard personal impactor sampler (Burkard Manufacturing Co., Ltd., Rickmansworth Hertfordshire, England) are described elsewhere (Buttner et al. 1997).

RESULTS

Epidemiological Investigation

A total of 557 of the 650 surveys were returned (86% response rate). The distribution of symptoms by floor was relatively constant, with a mean of 25% total case prevalence. Confirmed cases were mapped by geographic distribution on each floor. Cases were observed to be clustered around the perimeter of each floor, though not necessarily next to windows. When this graphic representation was superimposed over the building's engineering blueprints, the pattern closely matched the distribution of the VAV boxes on each floor.

Although the statistical analysis of the occupant health survey was limited in nature for the purposes of the investigation and excluded other available data, the findings of a relatively uniform distribution and consistent (14-35%) prevalence of symptomatic occupants on each floor, the consistent nature of the symptoms (principally respiratory, mucous membrane, constitutional and neurocognitive), and the significant number of (possible) cases involving aggravation of underlying allergic rhinitis or asthma were sufficient to formulate a hypothesis of a non-infectious, microbiological etiology with an allergic-type component.

Environmental Investigation

Based upon the results of the occupant survey, a walk-through evaluation was conducted which located previously undetected water damage (stains) to ceiling tiles beneath the VAV boxes throughout the building. Additionally, areas of the 2nd floor atrium wall, ladies restroom, and surrounding office areas were found to have evidence of water damage to sheetrock walls, and walls on the 5th floor were found to have significant water staining behind wallpaper resulting from rooftop leakage.

A total of over 50 water-stained ceiling tiles beneath VAV valves were removed throughout the building. On the occupied side of the tiles, only a water stain was visible, but on the plenum side many of the tiles demonstrated visibly active fungal growth. Transparent tape sampling was conducted on water-stained ceiling tiles from selected areas on the 2nd floor. Tape samples and bulk sample cultures of the occupied side of tile samples identified colonization by *Alternaria*, whereas *Stachybotrys chartarum* was observed on the plenum side. Tape samples from the ceiling plenum above a 2nd floor women's restroom and atrium wallboard (non-occupied side) also revealed *Stachybotrys* spores and conidiophores. Visual observation of the water-stained 5th floor sheetrock beneath wallpaper revealed visible mold growth. Tape samples also

confirmed the presence of *Stachybotrys* spores and conidiophores, as well as spores and hyphae of *Chaetomium* and *Alternaria*.

Andersen sampling in select locations on the second floor revealed few airborne culturable fungi and Burkard air sampling was negative for *Stachybotrys* spores. Swab samples of roof-top AHU supply ducts and fans, supply registers, and fire damper liners obtained on the second floor, isolated few, if any, culturable fungi.

Engineering Evaluation

The hot water valves which supply the VAV heating coils were found to be the source of the moisture to the ceiling tiles. Approximately 40% of the 250 VAV hot water valves in the building revealed evidence of past or present water leakage, as manifested either by a stained ceiling tile below, or rusting or discoloration of the metal components of the valve. The two "O-ring" seatings on all of the leaking valves were found to be significantly worn for their age. Water stains on the atrium wall and in the ladies room pipe were attributable to unrelated, localized plumbing leakages which were not found to be prevalent elsewhere in the building. The water-damaged sheetrock on the 5th floor appeared to be the only occupied area damaged by leakage from the roof and a single, isolated drainage obstruction from the rooftop was identified as the cause.

Exposure Intervention

All water-stained ceiling tiles were removed while the building was unoccupied. Area containment was not utilized. The tiles were sealed in plastic bags and transported off-site. All VAV hot water valves were tightened, and defective O-rings were replaced. (The specific cause for the defective seatings remains under investigation with the manufacturer and building contractor). Other identified areas of water-damaged building materials were removed and replaced. A program for ongoing maintenance surveillance of ceiling tiles was implemented by the building maintenance staff.

Within four (4) weeks of these interventions, occupants' complaints (verbal and by e-mail) subsided substantially. A formal follow-up occupant survey was not performed due to logistical and cost constraints.

DISCUSSION

This case study describes the outcome of an investigation of previously unexplained, chronic, building-related health complaints among occupants of a modern office building that had been ongoing for more than two years since the building opened. The evidence that a unifying, scientifically plausible etiology of the health problem was microbiological - and specifically, the colonization of water-damaged ceiling tiles throughout the building by *Stachybotrys chartarum* and *Alternaria* - was provided by a combination of epidemiological, microbiological, and physical evidence. Exposure to *Chaetomium* spores was also a potential source of illness, though this contamination was confined to just one (5th) floor of the building. The route of occupant exposure to fungal spores was theorized to occur through airborne migration of dessicated spores from the plenum to the occupied spaces when the latter became negatively pressurized at night

when the ventilation system was dormant. The limited air sampling which was conducted may thus have missed detection of culturable fungi in the air. Nevertheless, the direct association between mold contamination of ceiling tiles and chronic, building-wide illness was confirmed by the rapid, uniform response of the occupants' symptoms to removal of the contamination problem.

The identification of a common source of ongoing water damage to building materials, and the underlying cause of this previously undetected water damage, was critical in validating the microbiological explanation for this "sick" building. Most likely this problem started before the building was opened for occupancy. The finding that 40% of the valves had leaked suggested that the leakage was an intermittent, building-wide problem. Improperly sized O-rings, defective or incorrect rubber or polymer used in their manufacture or application, or the use of an incompatible corrosion inhibitor in the VAV hot water valve system are plausible explanations for the ubiquitous valve leakage. In the presence of a continuous or intermittent (warm) water source and low-nitrogen cellulose ceiling tile material, fungal colonizers such as *Stachybotrys* and other hydrophilic fungi can thrive indefinitely, or sporulate and grow in cycles as the water evaporates and the surface desiccates, followed by another episode of leakage. Potential contributory sources of dissemination of spores to the occupied space include normal mechanical vibration of ceiling tiles, vibration from the operation of the VAV fans, and temporary negative pressurization of the occupied space relative to the plenum during periods when the VAV in an area is non-operational.

Stachybotrys chartarum and other toxigenic fungi are becoming increasingly associated with building-related illnesses (BRI) and SBS in other published cases of IAQ building investigations ((Johanning 1996; Harrison et al. 1992; Sorenson 1987). It is possible that potential microbiological sources of contamination were overlooked in previously published and unpublished "sick building" cases that were investigated and labeled with "psychogenic" or "multifactorial" etiologies (Skov et al. 1989; Stenberg et al. 1994). The potential mechanisms of disease due to this and other toxigenic fungi is currently the subject of active research (Fung et al. 1998). Trichothecene mycotoxins produced by these fungi have been identified in the dust of ventilation systems of "sick" office buildings (Smoragiewicz et al. 1993). Additional SBS investigations have identified other colonizing fungi, such as *Chaetomium* which was identified in wet sheetrock in this building, and certain *Penicillium* and *Aspergillus* species (Bernstein et al. 1983), among many others, in water-damaged building materials (Sudakin 1998; Hodgson et al. 1998).

Practical constraints of time and financial resources for more rigorous statistical, microbiological, and exposure assessment measurements were a limiting factor in providing more "objective" evidence of routes of exposure and disease, as well as follow-up evaluation. The case definition employed in the occupant survey analysis was intentionally designed to be sensitive rather than specific because the study was employed primarily to estimate disease prevalence and distribution, to form a differential diagnosis, and to guide the environmental and exposure assessment. The occupant health study was subject to misclassification bias (overdiagnosis of cases) due to the component of subjective decision-making required, and the reliance upon a single, logistical question for case definition. Occupants could conceivably over-report symptoms to validate their concerns about building-related health problems and concerns. The results suggest that these sources of bias were minimal. The currently available immunological

tests (e.g., IgE and IgG *Stachybotrys* antibodies) used as markers of exposure or disease related to indoor mold exposure are of low specificity and positive predictive value (Hodgson et al. 1998; Johanning et al. 1996; Malkin et al. 1998). For this reason, as well as cost and logistical considerations, these diagnostic tests were not employed in confirming or detecting the microbiological etiology.

The finding of negative air sampling for culturable fungi (spores) on a single sampling date should not negate the significance of the finding of active fungal growth on ceiling tiles throughout the building. Release of spores from certain hydrophilic fungi, including *Stachybotrys chartarum*, is known to be intermittent and subject to various physical and mechanical factors (Flannigan 1994). Additional sampling in various locations at various times of the day would probably have helped to confirm the route of exposure of spores from the plenum to the occupied areas and breathing zones of occupants.

CONCLUSION

An epidemiological investigation of occupants' symptoms in a "sick" office building and a subsequent, focused microbiological investigation led investigators to correctly diagnose and remediate previously undetected water damage to and fungal growth on building materials that was consistent with occupants' health complaints. The implementation of a formal epidemiological methodology served to properly define the nature, extent, and validity of the clinical illness, which had previously been dismissed after a limited medical and air quality evaluation. The identification of a common source of ongoing water damage to building materials, and an underlying cause of this previously undetected water damage, was critical in validating the microbiological explanation for this "sick building." Other cases of "sick buildings" may have similar, unrecognized sources of microbiological contamination which would benefit from this investigative approach.

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FUNGAL EXPOSURE AND IgG-LEVELS OF OCCUPANTS IN HOUSES WITH AND WITHOUT MOLD PROBLEMS

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ABSTRACT

The aim of the study was to evaluate the relevance of serum antifungal-IgG antibodies to reflect the individual's exposure to building related fungi. The concentrations of viable fungi were higher and composition of fungal flora was different in the index houses with moisture problem compared to those in the reference houses. Positive IgG-findings were common in both groups, although higher antibody levels against most of the fungi were found in the study group than in the control group. However, compatibility between fungal flora found in each house and elevated IgG-levels of its occupants were seldom found in either of the groups. This study suggests that microbial sampling gives information of the building at the time of the measurements, whereas IgG-antibodies reflect long-term integrated exposure of an individual from total environment including the work place.

KEYWORDS:

Fungal exposure, IgG antibodies, mold problem buildings

INTRODUCTION

In working environments, such as sawmills and agriculture, the presence of microbe-specific IgG antibodies has been shown to reflect exposure to such antigens (Eduard 1993, Erkinjuntti-Pekkanen 1996). The aim of this study was to evaluate the relevance of serum antifungal-IgG antibodies to reflect an individual's exposure to building related fungi by determining the fungal flora in private houses with moisture and mold problems and in reference houses and IgG-levels of their occupants.

MATERIAL AND METHODS

THE BUILDINGS STUDIED

The study was carried out in twelve buildings with moisture or mold problems (index) and twelve buildings without any such problems (reference). All the index buildings had a history

of water damage and microbial growth on the surfaces of building materials, which was confirmed during site visits. In the reference buildings no signs of such problems were found.

Subjects

IgG-levels were determined from the sera of 25 individuals living in the index houses (study group) and 17 individuals living in the reference houses (control group).

Sampling and analysis

The fungal concentrations and flora in the houses were determined with air and surface samples, additionally, building material samples were analyzed in the index houses. The samples were taken or plated on malt extract agar (MEA) or 2% malt extract agar (M2) and incubated at 25°C for 7 days. Fungal colonies were identified using optical microscope.

Air samples were taken with a six-stage impactor (Andersen 10-800) in 2-6 different rooms in each building in the winter. The sampling time was 10 min. Surface samples were taken with a sterile swab into sterile Tween 80 dilution water and a series of dilutions were prepared and plated on the media. Sampling area was 100 cm^2. Building material samples were taken from visibly contaminated sites. The samples were weighed, homogenized and extracted with sterile Tween 80 dilution water. Suspensions were held in a ultrasonic bath for 30 minutes and in a shaker for 60 minutes. Dilution series were made and plated on the media.

Antibody determination

On the basis of the results from environmental sampling, twenty- four fungal strains were selected for antibody determination. Fungi were cultured in Roux bottles on mycological peptone broth (2% malt extract, 1% mycological peptone, 4% glucose in sterile water) for 7 days and then washed three times with phosphate-buffered saline (PBS), pH 7.4. After autoclaving, disrupting by a homogenizer and an ultrasonic treatment, microbial homogenates were centrifuged. After the filtration through 0.4μm pore size filter the supernatants were stored at 70°C until used as antigens in the enzymelinked immunosorbent assay (ELISA). Alkaline phosphatase-conjugated antihuman IgG (Sigma) was used in ELISA as antiserum for the detection of IgG antibodies in the serum samples.

In ELISA, the absorbance value of a test serum is compared to that of the pooled positive serum which is used as a control. The proportion of the absorbance given by the test serum from the absorbance given by the control serum is expressed as a percentage (%). In interpretation the ELISA results, these percentages are compared to the reference percentage values which were calculated from the sera of 200 volunteers, whose exposure to the antigens was not examined. Based on these values, we set the limits for the different exposure classes: negative or very low IgG-level is interpreted as an indication of normal environmental exposure to the corresponding antigen; low IgG-level is an indication of slight immunoactivation caused by the corresponding antigen; moderate IgG-level is indication of moderate immunoactivation, and high IgG-level indicates strong immunoactivation by the corresponding antigen.

RESULTS

MICROBES

In indoor air, the total concentrations of viable fungi in the index houses varied from 5 to 54 000 cfu/m^3 while the concentrations in the references were significantly lower ($p<0.001$) varying from 5 to 400 cfu/m^3. The concentrations of airborne *Penicillium*, *Aspergillus* and yeasts were also significantly higher in the index houses than those in references. In surface samples, the total concentrations of viable fungi ($p<0.001$) and the concentrations of *Aspergillus* ($p<0.01$), *Cladosporium* ($p<0.05$), *Acremonium* ($p<0.001$) and yeasts ($p<0.01$) were significantly higher in the index houses than those in the reference houses.

ANTIBODIES

Positive IgG-findings were common in both groups, although higher antibody levels against most fungi were found in the study group than in the control group. However, compatibility between fungal flora found in each house and higher IgG-levels of its occupants were seldom found in either of the groups. In the control group, high IgG-levels against *Trichoderma*, *Humicola*, *Stachybotrys* and *Rhizopus* were clustered to a few persons with no evident exposure at their homes.

DISCUSSION

Microbial sampling gives information of the building at the time of the measurements, whereas IgG-antibodies reflect long-term integrated exposure of an individual from total environment including the work place. It is possible that the present fungal flora found in the moisture damaged buildings are a result of microbial succession and hence those microbes that have already made way for other microbes are no longer necessarily found. Some of the microbes indicating moisture, e.g. *Phialophora* (Samson et al. 1994) were found only in the reference houses; thus the boundary between the study groups might be obscure. The high IgG-levels found in the control group are likely from earlier and already ceased exposures or from other places than home. This indicates that IgG-levels of an individual do not show where and when microbial exposure takes place.

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THE IMMUNOPATHOLOGY OF HYPERSENSITIVITY REACTIONS

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ABSTRACT

Overactivity of the immune system, either allergy (IgE) or hypersensitivity (non-IgE) is responsible for more illness than is generally appreciated, even by the medical profession. The least understood are the non-IgE mechanisms which involve either immune complex formation (type III of Gell and Combs) or direct killer T-cell involvement (type IV). Type III reactions may be localized with a large deposition of antigen at a focal point where immune complexes are formed and tissue damage ensues including necrosis. This is termed the Arthus reaction. A systemic dissemination of antigens will provoke a systemic inflammatory reaction which is most closely modeled by the well studied acute and chronic serum sickness reaction. Serum sickness was identified as the constellation of symptoms which followed the administration of antitoxins (antisera given for infectious disease before the advent of antibiotics) which were derived from non-human sources, most often horses. Chronic serum sickness was observed when otherwise healthy subjects were given repeat doses of antisera experimentally over relatively short periods of time. The symptoms observed in spontaneous and experimental serum sickness included fatigue, rash, cognitive changes, myositis, arthritis, headache, weight-loss, cardiovascular symptoms etc., which are often seen during heavy chronic exposure to fungal spores. The dynamic nature of circulating immune complexes, their complexity, their rapidly changing exquilibrum patterns and their pathogenicity must be appreciated before the clinician can properly interpret the patterns of illness his patients' describe. The best simple test identifying and thereby allowing the avoidance of serum sickness is a specific IgE test to a panel of high exposure antigens including fungi, food and occupational antigens.

KEY WORDS

Mechanisms of Hypersensitivity, Immune complexes, Immunopathology of fungal reactions

There is considerable controversy about the potential health hazards from exposure to fungal elements which is fueled by the growing amount of litigation involving this issue. Workers, homeowners, students who become ill, identify the potential source of symptoms as a contaminated environment and sue their companies, home builders or school authorities respectively for compensation. The litigation process with hired "expert" testimony from both sides of the controversy does little to add to our knowledge of the health hazards or safety limits on exposure to fungi. Health care professionals have to be aware of the potential for illness fol-

lowing exposure to fungi and sufficiently alerted to the possibility of a fungal cause of the illness to arrive at a correct diagnosis and thereby help their patients.

There are three commonly recognized sources of fungal exposure, which have the potential to produce disease. These come via airborne routes in the form of spores, volatile compounds and l fragments; ingestion as a food contaminant or food additive or by-product of food processing; and via direct contact from colonization of skin, mucosal surfaces and the alveoli of the lungs. Since different fungi from different sources share common surface proteins, the immune response to fungi may crossreact and avoidance measures in treating hypersensitivity reactions must identify and correct all sources of contact.

Mycotoxins are the most respected of fungal products for their potential to cause serious illness through their direct biochemical action on key body functions. Aflatoxin is known to be among the most potent of carcinogens and the toxin released by *stachybotris* is believed to have caused deaths in exposed infants. Other toxins can affect various hormonal, neurological and other body functions to produce serious health effects. They are so effective in certain biological activities that they have been harnessed by the pharmaceutical and food industries to commercial use such as antibiotics, immune suppressants to control graft rejection, cholesterol control, and enzymatic activity in food processing and preservation. Mycotoxins are produced by some fungi under specific growth conditions and are relatively rare causes of human illness. Hyperactive immune systems responding to the influx of fungal antigens following chronic exposures are much more likely to be a cause of symptoms in most individuals. Since hypersensitivity states develop only after relatively long exposure times, children under ten years of age do not have significant antibody titers to fungi unless they have experienced very high exposure levels, while normal mature adults living in temperate or tropical climates commonly show sensitivity to fungi and experience symptoms following unusual exposures. These symptoms often follow exposures by one or two days, are not recognized for what they are, and are likely to be diagnosed as a virus infection.

Allergists have accepted the role that fungal spores can play in eliciting allergy symptoms in susceptible individuals. This is akin to the effects of other airborne organic particles such as pollen, animal dander's and insect dust. The illness affects only individuals programmed genetically to make large quantities of specific IgE antibodies on exposure to relatively small amounts of allergen. This is a type I immunopathology as defined by Gell and Coombs (1) and involves the release of pre-formed histamine and other biologically active cytokines from sensitized mast cells and basophils. Symptoms include watery nasal discharge, sneezing paroxysms, itching of the naso-oro-pharyngeal mucosa and tearing eyes and can be significantly disabling. It has been suggested that perhaps five percent of the population may be affected in this way by fungi.

Far more elaborate and damaging immune responses can be elaborated by the body following exposure to large amount of fungal particles, especially when the exposure is chronic. These illnesses were originally described in association with various occupational exposures in unprotected workers such as farmer's lung, bagassosis in sugar cane workers and many others. More recently such conditions have been identified and studied in office workers whose workplace is contaminated by fungi, especially in buildings with closed ventilation systems. The disease has also been described in individuals exposed to swamp coolers or contaminated air condi-

tioners in the home. These symptoms can occur in all individuals with normal immunity because they are ultimately manifestations of a robust immune response to a heavy airborne fungal load with consequential overload of clearing mechanisms or macrophages, and the activation of inflammatory processes.

The mechanisms of disease produced by heavy, chronic exposure to airborne fungal elements are classified as Types III and IV immunopathologys (Gell and Coombs) and involve antibodies, primarily IgG, and activated, sensitized lymphocytes. The best single marker of such an immune response is specific serum IgG which can help identify the fungal causes of the illness and lead to remediation to remove fungal sources of exposure. The only known treatment for the condition is cessation of exposure. Once the hypersensitivity has developed, all sources of fungi must be removed including treatment of colonization and a mold-free diet.

The spectrum of symptoms produced by exposure of healthy, normal individuals to heavy doses of fungal elements varies from throat or nasal irritation to severe incapacitating influenza-like illness with malaise, rash, muscle soreness, joint stiffness and pain, fever, night sweats, weakness, headaches, and mental changes including cognition. In the patient with diminished resistance such as the very old or very young, the chronically ill such as patients with diabetes, patients with suppressed immunity as a result of cancer chemotherapy or organ transplantation; and patients with AIDS, certain fungi will invade the body with disastrous consequences including death.

When foreign materials (antigens) enter the body's tissues for the first time a series of events is initiated which leads to the formation of specific antibody which combines with the antigen and leads to its rejection from the body by the action of scavenger cells or macrophages. The antigen-antibody unit is called an immune complex and it is rapidly taken up by the macrophages under normal circumstances. When the antigen is a virus, the viral-antibody complex is removed from the circulation, the infection is over leaving a temporary state of immunity. If the antigen is a fungal spore continually being broadcast into the air by a contaminated air conditioning unit, the "infection" never stops. The immune state is repeatedly or continually challenged to cope with large numbers of foreign invaders. The macrophage system can become saturated and complexes can no longer be safely removed from the circulation. The complexes may be then removed by tissue deposition into the skin, blood vessel walls and specific organs such as the lungs or kidneys leading to rashes, vasculitis, alveolitis (lungs) and glomerulo-nephritis (kidneys) respectively.

The behavior of circulating immune complexes is determined by several semi independent variables such as absolute and relative concentrations of antigen and antibody in the circulation, the solubility of the complex, and its size and surface charges. There are three important antigen-antibody equilibria which determine the pathogenicity of the complex and the clinical course of the exposures. In antigen excess the stable complex consists of an antibody with an antigen at each of its two binding sites. At antibody excess the stable complex consists of an antigen with a different single antibody at each of its recognized epitopes or antibody reactive sites. At more equal concentrations of antigen and antibody, the complexes are at equivalence and can grow to enormous size. The most pathogenic equilibrium is near equivalence. Because of the nature of the antigen-antibody binding site, the complexes can easily shift from one equilibrium to another as relative concentrations of antigen and antibody

shifts. This complexity in the disposition in the body makes it difficult to study the phenomena and even more difficult for the clinician to understand its ever changing effects on the patient.

Most patients who are experiencing symptoms from exposure to environmental fungi exposure have become hypersensitive and are usually in the antigen excess side of equivalence at the time they seek medical care. Removing the source of exposure may temporarily (one to two weeks) lead to an increase in symptoms as the patient's moves through equivalence and into antibody excess. At this time, even small exposures produce exaggerated symptoms because of the move into equivalence. The patients must be forewarned of this phenomena before it happens because it increases compliance.

If the patient has had previous infections involving exposed surfaces which have caused tissue damage or scarring, the possibility of fungal colonization increases. This would include chronic sinusitis, pneumonia or gastroenteritis. In these cases, a fungal component of the disease must be suspected and a therapeutic trial of an antifungal may be necessary. Nystatin, econazole and miconazole are poorly absorbed and are effective in treating colonization of the G.I. tract. The more soluble anti-fungals itraconzole, terbenafine and fluconazole may be helpful in reaching the lungs, nails or sinuses.

Treating a hypersensitive (to fungi) patient who is heavily colonized with a fungus (e.g. *aspergillus*) with an effective antifungal (e.g. itraconzole) will almost always produce a Jarisch-Herxheimer (J.H.) reaction due to the inability of the bodies clearing mechanisms to dispose of the surge in immune complexes following initiation of therapy. It is, therefore, prudent to begin treatment with low doses of antifungal and progress upward every few days to a week as the patient tolerates the therapy. The symptoms of a J-H reaction are flu-like with malaise, fever, headache, gastroenteritis, myositis, nasal congestion, CNS disturbances, etc. When they occur, the treatment should be temporarily interrupted to allow the patient to recover, and then resumed at the last tolerated dose for a few weeks before trying to increase the dose again. J-H reactions can occur at any stage as the dosage is being increased and may occur after therapeutic levels are safely reached should there be a sudden increase in ambient fungal levels or following ingestion of a food containing a significant fungal component.

The quantity of fungal elements in foods is not appreciated by the general public, by nutritionists, and even by physicians specializing in the treatment of food allergy. Only the food scientists really know the full extent of fungal usage in foods and they often do not show this on package labels. Chocolate, tea and soy sauce are fermented by *aspergillus* species. The Lactase used to remove lactose from milk for milk-intolerant patients is an *aspergillus* product. Malt extracts, dough conditioners and amylase used in baking are all of fungal origin. Most commercial fruit juices are not only extracted from moldy fruit but have had fungal cellulases added to the pulp to increase yields. Citric acid, a major acidulant/preservative used in processed foods is an *aspergillus* fermentation product, not a lemon extract. All tomato products are laced with fungal derivatives, and of course, all foods become moldy in time and frequent grocery shopping is essential to a mold-free diet.

The best studied human model of immune complex-mediated illness following chronic exposure to absorbed antigen is chronic serum sickness. There is considerable experimental data on this subject published in the middle third of this century (2). This work was, of course, stim-

ulated by the adverse effects of horse serum (antitoxin) administration for the treatment of diphtheria and tetanus, but the elements are the same as seen in fungal hypersensitivity disease. Figure II lists the common clinical symptoms seen in serum sickness and demonstrates how widespread are the possible adverse effects one can expect from circulating immune complexes.

Circulating immune complexes containing IgG or IgM antibodies are highly inflammatory acting on serum complement which triggers the activation of a cascade of enzymes which play various roles in the destruction and removal of foreign materials from the body. Two of these components, C'3 and C'5, are known to cause the release of histamine from mast cells thus mimicking the Type I allergy reaction although delayed in time from minutes for the IgE activation to hours for the C'3, C'5 action. Because of the time requirement for antigen uptake, complex formation, macrophage overload and tissue deposition, Type III hypersensitivity reactions are delayed by hours or days from the time of exposure. This makes it more difficult to appreciate the cause-effect relationship of exposure to illness. Type IV cellular reactions are similarly delayed in onset.

By understanding the nature of a hypersensitivity reaction, its multiple interactives, it's delayed onset and the widespread nature of its inflammatory potential, an extremely complicated system can be better appreciated. The underlying principles can be applied to a better understanding of human hypersensitivity diseases caused by exposure to relatively benign, common, non- mycotoxin producing fungi.

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EXPOSURE TO STACHYBOTRYS CHARTARUM INDUCES IMMUNOGLOBULIN A ANTIBODY RESPONSE IN MAN

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ABSTRACT

The levels of serum immunoglobulin (Ig) E, G and A antibodies against *Stachybotrys chartarum* were measured in patients with asthmatic or mycotoxicosis symptoms (n=6), their family members (n=10) and control subjects (n=18) with indirect enzyme-linked immunosorbent assays. The *Stachybotrys*-specific IgG and IgA levels correlated with each other significantly ($r=0.71$, $p<0.01$) in the group of exposed subjects (patients and family members), but not in the control group. The IgA levels were significantly higher ($p<0.01$) and the IgG levels slightly higher ($p<0.05$) in the patient group than in the control group. IgE levels did not differ between the subject groups. The results of this study suggest that the exposure to *Stachybotrys* does not cause IgE-mediated allergy in humans. It is possible that the IgA response reflects better exposure to the fungus than the IgG response does.

KEY WORDS

Serum antibody level, *Stachybotrys chartarum*, exposure, enzyme-linked immunosorbent assay, IgA, IgG, IgE

INTRODUCTION

Exposure to airborne fungi is known to cause adverse health effects e.g. irritation of the respiratory tract and the eyes, cough, wheezing, headache and respiratory infections. Besides, several fungi are common allergens and cause allergic rhinitis, asthma and allergic conjunctivitis (Husman 1996, Fink 1998, Reijula 1998). Serum immunoglobulin (Ig) G antibody levels against specific fungal antigens are regarded to be related to exposure to these micro-organisms and are often used as biomarkers of exposure in the working environment (Eduard 1995). For the diagnosis of mould-induced allergy, the demonstration of IgE reactivity to fungal antigens is important (Fink 1998). About 5-30 % of the asthmatic population seems to be allergic against moulds depending on the species tested (Mailing 1992). *Stachybotrys chartarum* is considered to be one of the moisture-indicating moulds in water-damaged buildings (Samson et al. 1994). Because of its ability to produce mycotoxins (Jarvis 1991), *S. chartarum* is especially hazardous. Beside, *S. chartarum* is allergenic (Johanning et al. 1993) and it has also been reported to trigger

histamine release by non-IgE-mediated mechanisms (Larsen et al. 1996). In this study, the immunological responses of exposure to *Stachybotrys* were investigated by measuring the levels of *S. chartarum*-specific IgE, IgG and IgA antibodies in the sera of exposed and control subjects.

MATERIALS AND METHODS

Preparation of the antigen extract

Stachybotrys chartarum (UKU 10) was cultured on 2% Malt extract broth in stationary Roux bottles for 10 days at 25°C. Mycelium (containing spores) was dried in a vacuum dryer and resuspended at 1:20 (w/v) in PBS (pH 8.0) with 0.02% (w/v) phenylmethyl sulfonyl fluoride and 0.02% (w/v) natrium azide. Mycelium suspension was homogenized and extracted in a shaker for 16-18 h at 4°C. After centrifugation, the supernatant (culture extract) was dialyzed (molecular weight cut-off 3.5 kD) against PBS at 4°C for 24 h, sterile-filtered (0.2 µm) and lyophilized. Lyophilized extract was stored at -70°C and resuspended in MilliQ-UF water for use.

Subjects

A group of patients with asthmatic or mycotoxicosis symptoms (n=6), their family members (n=10) and control subjects (healthy students, n=18) were studied. The patients and their family members were suspected to be exposed to *Stachybotrys* in their homes according to the environmental analyses and/or symptoms.

Enzyme-linked immunosorbent assay

The levels of IgE, G and A in human sera were analyzed with the indirect enzyme-linked immunosorbent assays (ELISA). Microtitre plates were coated with the *S. chartarum* extract at a protein concentration of 5 µg/ml for IgE-analysis and 1 µg/ml for IgG and IgA analyses as described by Virtanen et al. (1988). 100 (100 µl of human sera, at dilutions of 1:10, 1:400 or 1:25, respectively, were added in duplicate into the wells of the plates and the plates were incubated at 37°C for 1 h. The diluent, used also for washes, was PBS, pH 7.4, containing 0.1% casein (w/v) and 0.5% (v/v) nonionic detergent, Nonidet P40. After three washes, 100 µl of rabbit anti-human IgE, peroxidase-conjugated rabbit anti-human IgG or peroxidase-conjugated rabbit anti-human IgA were added at dilutions of 1:500, 1:2000 or 1:2000, respectively, and incubated at 37°C for 1 h. Rabbit anti-human IgE was detected with biotinylated goat anti-rabbit IgGs (1:1000, 1 h at 37°C) followed by Vectastain Elite ABC reagent (1:500). Color reactions were developed and measured as reported by Virtanen et al. (1988). Differences in the Ig levels between the subject groups were tested with the Mann Whitney U-test and the correlations with the Spearman rank correlation test.

RESULTS

The levels of IgE, G and A of patients, their family members and control subjects are presented in Table I. Among all the subjects, the *Stachybotrys*-specific IgG and IgA levels correlated with each other (r=0.34) but the correlation was not significant (p>0.05). The correlation

was significant ($r=0.71$, $p<0.01$) in the group of the exposed subjects (patients and family members). The IgA levels ($p<0.01$) and the IgG levels ($p<0.05$) were higher in the patient group than in the control group. The patients' IgA levels were also higher than those of their family members ($p<0.05$) while the IgG levels did not differ statistically between the groups. No statistically significant differences were found in the IgA and IgG levels between the family members and the control group, either. The IgE levels did not differ between any subject groups.

TABLE I: The mean levels (optical density measured by the ELISA, ±SD) of serum IgE, IgG and IgA of the patients (n=6), their family members (n=10) and control subjects (n=18).

	Patients	**Family members**	**Controls subjects**
IgE	1.02 ± 0.57	1.03 ± 0.53	0.95 ± 0.74
IgG	0.98[1] ± 0.48	0.53 ± 0.34	0.56[1] ± 0.39
IgA	0.84[2,3] ± 0.34	0.45[2] ± 0.17	0.46[3] ± 0.25

[1,2]$p<0.05$, [3]$p<0.01$

DISCUSSION

Johanning et al. (1996) measured the serum IgE, IgG and IgA levels of subjects exposed to *Stachybotrys* at their workplace. Only 4% of the employees had a positive IgE test for *S. chartarum*. Nor were the other Ig levels of the employees significantly elevated compared to the control group. At this study, no difference was found in the IgE levels between the patients and the control group either, but the patients' IgA level was statistically significantly higher than that of their family members and control subjects.

Serum IgG level is often used as a marker of a fungal exposure in the work environment (Eduard 1995). In some severe lung diseases like allergic bronchopulmonary aspergillosis (ABPA), the specific serum IgG and IgE levels have correlated well with the state of the disease (Kauffman et al. 1983; Knutsen et al. 1994). At this study, the IgG levels were also higher in the patient group than in the control group.

IgA antibodies prevent the attachment of bacteria or toxins to epithelial cells or the absorption of foreign substances, and provide the first line of defense against a wide variety of pathogens (Janeway and Travers 1997). To our knowledge, there are only few studies concerning the effect of the fungal exposure on serum IgA levels. Apter et al. (1989) reported that the level of the serum IgA often rose before or during the exacerbation of ABPA and that the fluctuation of the IgA level in serum was similar to that of IgE level. Sennekamp et al. (1990) reported that the serum IgA level was a more specific factor for indicating farmers lung, a disease associated with fungal exposure, than the IgG level, and Ojanen et al. (1990) made the same conclusion about the antibodies against *Aspergillus fumigatus*. Yoshikawa et al. (1995) reported that when specific IgA was detected in the patient sera the IgA concentration in the bronchoalveolar lavage was very high, which suggests that the concentration of the serum IgA may depend on the magnitude of respiratory exposure to fungi.

In conclusion, our results supports the view that IgA response reflects better the inhalation exposure to *S. chartarum* than IgG response does.

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IMPACTS OF AIRBORNE VIRUSES ON INDOOR ENVIRONMENTS

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ABSTRACT

The presence of airborne viruses in the indoor environment can lead to a significant incidence of occupant morbidity. Although many of the illnesses caused by airborne viruses are relatively benign or self-limiting, there are strains of viruses, e.g. influenza, which can result in mortality. Certain populations, such as immunocompromised, are especially at risk. Respiratory disease accounts for approximately 75-80% of all acute morbidity in the U.S. with nearly 80% of the illnesses viral (Ray 1994).

In the U.S., we spend over five billion dollars annually on remedies for relief of the common cold caused, primarily, by rhinoviruses (Radetsky 1991). The common cold is also responsible for an aggregate of thirty million sick days from work and school due to its debilitating effects (Radetsky 1991). The principle modality for transmission of the common cold is via an airborne pathway (Dick and Inhorn 1992).

Properly designed, implemented, and operated engineering control strategies may be an effective intermediate term approach to breaking the chain-of-transmission of illness attributed to respiratory viruses. This paper reviews some background on the prevalence of airborne viruses in the indoor environment, currently available engineering control strategies, and research needs in the area of indoor environmental virology.

KEY WORDS

Viruses, indoor environment, ventilation, common cold, airborne transmission

BACKGROUND

In recent years, an increasingly concerted effort has made to gain a better understanding of the health effects that indoor air contaminants have on occupants. Much of the research has focused on single and multi-component species of volatile organic compounds (VOCs), bacteria, and fungi. Individuals exposure to VOCs in indoor environments can lead to sick building-type symptoms or undesired sensory irritation (Alarie, 1981 and Schaper, 1993). The presence of bacteria in indoor environments has been linked to significant respiratory-related illnesses such as Legionnaires disease, Pontiac fever, and tuberculosis. Certain species of fungi are responsible for the production of toxic by-products that result in significant health risks to occupants with prolonged exposures (Johanning et al. 1996; Dearborn et al. 1994; Flannigan and Miller, 1994.).

There has been much less emphasis placed on conducting investigations that focus on understanding impacts of airborne viruses in indoor environments. Respiratory illnesses, attributed

to viruses, are a significant cause of morbidity annually. This paper reviews some of the data on the impact of viruses on the indoor environment and identifies a number of research needs to mitigate the incidence of disease caused by airborne viruses in indoor environments. Ultimately, the development of approaches to successfully control the concentration or existence of these agents in the indoor environment will have a significant and positive impact toward improving occupant health.

Viruses

It has happened again. Someone in your office has come down with a cold. After a day of sniffling, sneezing, and coughing, the infected co-worker has succeeded at dispersing billions of viruses (in the form of droplet nuclei) into the air in your office. Within a couple of days, you begin to feel rundown and, ultimately, experience the symptoms similar to those your recovering office partner felt in days prior. As the illness runs its course, you will likely miss one or more days of work as well as spend money purchasing cold remedies to aid in relieving symptoms of this dreaded "bug". This scene is played out regularly in indoor environments that include: office buildings, public transportation, healthcare facilities, schools, and daycare facilities. The viruses (airborne) responsible for several forms of respiratory illnesses include influenza virus, parainfluenza virus, rhinovirus, respiratory syncytial virus, and respiratory coronaviruses. The economic impact attributable to viruses that cause the common cold, e.g. rhinovirus, alone is staggering.

Viruses in the rhinovirus family are the source of more than half of common colds experienced by humans. Although disease experienced by infected individuals is usually self-limiting and relatively short in duration, the common cold exerts a significant economic impact worldwide. In the U.S. alone, we spend over five billion dollars annually on relief remedies (Radetsky 1991). The common cold is also responsible for an aggregate thirty million sick days from work and school due to its debilitating effects (Radetsky 1991). Establishing effective control strategies for rhinovirus infections should remain a top public health priority in the United States. However, due to the large number of unique rhinoviruses (over 100 serotypes have been identified - Dick and Inhorn, 1992), the implementation of effective prevention measures has been and will continue to be an enormous challenge. Since vaccination strategies have also proven impractical for this class of microorganisms due to their rapid mutation rates, the most plausible mitigation or control strategy appears to be through chain-of-transmission interruption.

Transmission of Viral Infections

Historically, two theories have emerged which characterize the modality for transmission of the common cold: airborne transmission and direct contact.

The Common Cold: Direct Contact Transmission of Viruses

Circa 1970, Gwaltney and Hendley undertook significant efforts to explore the hypothesis that colds are transmitted by direct contact. Their interest grew out of observations that rhinoviruses were inadvertently being spread to susceptible human subjects via. ethanol-sterilized nasal speculums during the course of their cold research (Dick and Inhorn, 1992). Hendley et al. (1973) later showed that one rhinovirus serotype, RV39, retained its infective characteristics for several hours when deposited on surfaces. They were also able to demonstrate that volun-

teers were capable of self-inoculation from environmental sources of RV39 by "nose-picking" and "eye-rubbing". Additional studies performed by the same researchers seemed to bolster the direct contact transmission hypothesis (Gwaltney and Hendley, 1978a; Gwaltney et al. 1978b).

As Dick and Inhorn (1992) point out, the Gwaltney et al. (1978b) transmission model was unrealistic when one considers the circumstances that existed during the course of their study. The study required infected volunteers to blow their noses into their hands. The infected donors then stroked recipient's hands for 10 seconds after which recipients inoculated themselves by placing a finger into their nose two or three times. This sequence of events yielded a 73% infection rate. Other independent researchers had a more difficult time achieving such a high infection rate under more plausible personal hygiene circumstances.

The Common Cold: Airborne Transmission of Viruses

Dick and his colleagues at the University of Wisconsin-Madison undertook a series of experiments to investigate the hypothesis that airborne pathways are responsible for transmission of colds. Dick's most notable and relevant studies are cited here.

Dick set-up mini-epidemics based on experiments he conducted in an isolated Antarctic community. In these "Antarctic Hut" model experiments (1986), Dick used RV16 to establish mini-epidemics in an isolated population. The experiments, conducted at the University of Wisconsin, consisted of 12-hour marathons of cardplaying by 20 students (eight donors and 12 recipients). Half of the recipients were prevented from self-inoculation with the use of hand restraints. The other six recipients were exposed (potentially) to viruses from the infected donors via bioaerosols and by indirect contact to contaminated fomites. Interestingly, the donors who were prevented from self-inoculation exhibited an infection rate (56%) which was very similar to that exhibited by recipients exposed to both airborne aerosols (from infected individuals coughing, sneezing, talking, etc.) and fomites (67%).

To further explore the potential for infection by the "direct-contact" route (hand-fomite-hand), another experiment was conducted where the aerosol pathway was interrupted (Dick et al. 1987). In this case, the eight infected students continued playing cards alone in an isolated room. Fomites deliberately infected with viruses (consisting of soggy playing cards, pencils, poker chips, etc.) were provided to 12 healthy recipients in another isolated room. The 12 new recipients played cards with the previously contaminated fomites and exaggerated fomite-hand-nose self-inoculation movements. Not one of the 12 recipients was infected! Dick concluded that the airborne route was the dominant pathway for the transmission of the common cold.

Independent of Dick, Agah et al. (1987) investigated the transmission of respiratory syncytial virus (RSV) spread from patients to nursing staff. Approximately half of the nursing staff used ordinary hygiene practices while the remainder used masks and goggles. For the staff using ordinary hygiene practices, 37 of 61 (61%) were infected while only 4 of 79 (5%) of the mask/goggle group were infected. These results appeared to strengthen the relationship between airborne transmission and respiratory disease.

CONTROL OF VIRUSES IN INDOOR ENVIRONMENTS

There have been several techniques and approaches developed that focus on disease prevention including: interrupt transmission, preventive treatment, identification and treatment of individual cases, immunization, and immunomodulation. Interrupting the chain-of-transmission has the potential of being an extremely effective approach to airborne viral-related disease prevention - in the near term. Transmission interruption relies heavily on the development and validation of engineering control strategies.

VENTILATION/ DILUTION

One approach to minimize the concentration of airborne pathogens in the indoor environment is increasing the quantity of outside air (ventilation air) delivered to the breathing zone of the occupants. The net result is a dilution of airborne pathogens in the indoor environment, leading to a probabilistic reduction in occupant morbidity. This methodology has been the primary engineering control approach used to-date as documented by ASHRAE (1989).

Ventilation/dilution has the advantage of being relatively simple in concept, but is unappealing due to its diminishing returns. As ventilation rates increase, operating costs increase significantly while the concentration of airborne pathogens decays asymptotically. The exponential decay shows that reducing the pathogen concentration by half requires doubling the ventilation rate. Since the fan power alone increases with the cube of the ventilation rate, doubling the ventilation rate has significant consequences in areas with extreme climates (warm humid, cold, etc.).

FILTRATION

The use of high efficiency particulate arrest (HEPA) filtration offers an effective approach for sequestering relatively large particles including many bacteria and fungi. HEPA filters are disposable extended-media filters capable of removing 99.97% of particles at a 0.3 μm aerodynamic diameter - the worst case diameter for filtration efficiency. Such performance may provide effective control of most airborne bacteria and fungi; however, mono-dispersed or droplet nuclei viruses may penetrate HEPA filters.

Filtration has the advantage of being a simple and static technology. Filtration has the disadvantage of increased capital costs, higher air-side pressure drop, increased energy costs (more fan power and fan heat added to supply air stream), and increased maintenance costs (filter replacements).

Ultraviolet Germicidal Irradiation (UVGI)

UVGI is capable of inactivating viruses and bacteria by exposure of a lethal ultraviolet radiation dose (combination of intensity and time). The biocidal properties of UVGI technology arise from interactions between ultraviolet radiation and genetic material in living cells (DNA). DNA exhibits a peak absorptance of electromagnetic radiation at a wavelength of 254 nm, well within the UV range. Prolonged UV exposure results in modifications to the internal structure of DNA, rendering it incapable of replication. UVGI is mentioned in CDC (1994) as an augmenting technology in control of *Mycobacterium tuberculosis*. There are organizations evaluating

the efficacy of UVGI specifically for TB control including the Centers for Disease Control, Harvard School of Public Health, Electric Power Research Institute, and others.

Although its biocidal properties for inactivating pathogens such as TB have been known for a number of years, its efficacy for a wide range of other pathogens (in air streams) including viruses and fungi have not been completely documented. An advantage of UVGI relates to its simplicity. The UV lighting technology is relatively mature; however, the application of the technology in the field is far from mature. UVGI suffers from the disadvantage of simultaneously increasing a facility's connected electrical load (kW) directly (through increased lighting) and indirectly (through increased cooling requirement). Additional disadvantages relate to significant maintenance/cleaning needs and ineffectiveness resulting from bioaerosols not being directly irradiated.

PHOTOCATALYSIS

A relatively new technology under current investigation is photocatalysis. Photocatalysis relies on the use of ultraviolet irradiation to activate a catalyst. The catalyst, once activated, produces OH- radicals capable of reacting with contaminants in the air stream. Ideally, the products of reaction are innocuous (i.e. carbon dioxide and water).

This technology has the advantage of potentially being effective in remediating a wide array of contaminants including viruses, volatile organic compounds, bacteria, and fungi. Photocatalysis has several disadvantages. First, most catalysts that have been developed to-date require relatively high temperatures (ca. 50ºC or higher) to achieve credible performance. This high temperature creates a dramatic parasitic load on HVAC systems. Second, most reactor designs have a very high air-side pressure drop (ca. 4 inches of water or higher). Third, this technology is capable of producing toxic by-products in situations where organic reactants have not been completely mineralized. Fourth, capital costs are projected to be very high since both catalysts and the UV light fixtures are expensive (Henschel, 1998). For these and other reasons, this technology is not expected to be competitive within the next 10 years.

RESEARCH NEEDS

The development and proof of effective strategies to interrupt the chain-of-transmission of airborne viral diseases will rely on conducting research to address a number of open issues related to indoor environmental virology. Some research issues that will provide a better understanding of indoor environment virus transmission characteristics, pathways, and technologies capable of interrupting the chain-of-transmission include:

- development of airborne isokinetic sampling devices capable of allowing enumeration of airborne viruses
- investigation on virus survival from the sampling processes
- confirmation of suitable media
- characterization of airborne viruses in the indoor environment: droplet nuclei size distribution,
- effects of "aging" on virus viability, changes in droplet nuclei size, and infectivity

CONCLUSIONS

Airborne viruses represent a significant source of pathogens in the indoor environment. Respiratory morbidity among indoor occupants results in a significant financial burden to those infected and, in some cases, results in mortality. Technologies exist today that can offer the potential to significantly minimize respiratory infections in indoor environments by interrupting the chain-of-transmission.

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SENSITIZATION TO MOLDS AND RESPIRATORY SYMPTOMS IN SCHOOL CHILDREN

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ABSTRACT

We performed a questionnaire study in 622 school children; asthma or asthmatic symptoms were present in 208 (33%) children. Moisture and mold problems were documented in the index school (N=414; 168 (41%) were symptomatic); the control school (N=208; 40 (19%) were symptomatic, $p<0.001$) had no such problems. Skin prick tests to 13 molds (9 occurring in environment and 4 indicating moisture problems in buildings) were performed in the 208 symptomatic children. A positive reaction (>3mm) was observed in only 5 (2%) children. Children who were exposed to molds in the school had more often emergency visits (OR=2.0, $p<0.01$) and also they had used more antibiotic courses (OR=2.1, $p<0.01$) than nonexposed pupils, but only during spring. Our results show that skin test positivity to molds is rare in school children. This is true in pupils with asthmatic symptoms, and also in the pupils from the school with moisture or mold problems. In addition, respiratory infections seem to be associated with mold exposure.

KEY WORDS:

Allergy, asthma, dampness problem, epidemiology, moisture problem, mold growth, mold exposure, sensitization to molds, respiratory manifestation in children, moisture problems in school buildings.

INTRODUCTION

Moisture problems in buildings easily lead to fungal growth in structures, further exposing the inhabitants to spores and other products of molds (Verhoeff et al. 1997). The association between moisture problems in buildings, sensitization to molds in children, and respiratory symptoms in sensitized subjects has been documented in epidemiological questionnaire studies (Munir et al. 1997, Taskinen et al. 1997). We performed a study including a questionnaire and skin prick tests in children from a school with technically and microbiologically documented moisture problems. The aim of this study was to evaluate the possible association between respiratory or allergic diseases among schoolchildren and moisture or mold problems in the school buildings.

MATERIALS AND METHODS

We performed a questionnaire study in 622 school children aged 7 to 13 years from two elementary schools, one being a moisture problem (index) school and the other a control school. A clinical study and skin prick tests (SPT) to 13 molds and 11 common allergens were done in 208 (33%) children with asthma, wheezing or cough; 168 (41%) pupils from the index and 40 (19%) from the control school belonged to this symptomatic group.

Both school buildings were investigated by trained building engineers, with special reference to moisture and mold problems, and microbiological studies were done in both indoor air and building material or structure samples.

Information on allergic diseases and asthmatic symptoms as well as on respiratory infections, antibiotic courses, emergency visits and hospital admissions were obtained by using a written questionnaire completed by the parents. The questionnaire comprised specific data on asthma and asthma-like symptoms, including repeated wheezing or prolonged cough.

Skin prick tests (SPT) were done in the 208 children who had had asthma, wheezing or cough symptoms during the preceding 12 months. The tests were performed with 11 common allergens and 13 molds; four of them (*Aspergillus fumigatus*, *Fusarium roseum*, *Phoma herbarum* and *Rhodotorula* rubra) were molds indicative of dampness.

RESULTS

The geometric mean of the total concentrations of the viable fungi was 21 CFU/m^3 in the index school being higher than that, 4 CFU/m^3, in the control school. The microbes indicating moisture problems, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Actinomycetes*, *Eurotium*, *Exophiala*, *Fusarium*, *Phialophora*, *Rhodotorula*, *Stachybotrys*, *Trichoderma* and *Wallemia* were found in indoor air and/or in building materials of the index school. *Aspergillus fumigatus*, *Actinomycetes*, *Eurotium* and *Wallemia* were also found in low concentrations in indoor air of the control school.

In the index school 168 children (41%) had suffered from either asthma, wheezing symptoms or prolonged nocturnal cough symptoms while in the control school the respective proportion was only 19% ($p<0.05$) (Table I). In the index school asthma was present in 4.6%, wheezing in 16% and cough in 21% of children. The figures in the control school were 5.3% for asthma (NS), 6% ($p<0.001$) for wheezing and 9% ($p<0.001$) for cough. The prevalences of atopic diseases were quite similar in the pupils from the index and the control schools.

Positive skin test reactions (>3mm) to molds were observed in 5 (2%) and weak reactions (1-2mm) in 8 (4%) children. There was a significant association ($p<0.001$) between reactions (strong and weak combined) to mold and asthma; 7 (25%) of the 28 children with asthma had such reactions, as compared to only 3% of those with wheezing or cough alone (Table I). No such association was found between mold sensitization and clinical atopy; that is, rhinitis, conjunctivitis or dermatitis.

TABLE I: Association between skin prick test results and the presence of respiratory symptoms and atopy.

Positive reaction in SPT	Asthma (N=28)	Wheezing (N=75)	Cough (N=105)	Atopy (N=108)
Pollens	71%***	40%	32%	57%***
Animal danders	71%***	36%	20%	51%***
House dust mites	25%*	11%	11%	12%NS
Molds[1]	25%***	4%	3%	9%NS
Moisture indicative molds[1]	11%*	3%	1%	4%NS

[1] weak and strong reactions combined. ***p < 0.001, *p <0.05 vs. children with no asthma or atopy respectively;

In the index school, emergency visits and antibiotic courses were more common in spring than in fall. This was not seen in the control school. During spring, children who were exposed to molds in the school had more emergency visits (OR=2.0, p<0.01) and used more antibiotic courses (OR=2.1, p<0.01) than nonexposed (Table II).

TABLE II: Emergency visits and antibiotic courses during spring 1995 and fall 1995 in the 457 pupils with data available.

Infection parameter	Index school (N=303)	Reference school (N=154)	Odds ratio	95%CI[a]
Emergency visit				
Spring 1995	31%**	19%[NS]	2.0	1.2-3.3
Fall 1995	20%	21%	1.0	0.6-1.6
Antibiotic courses				
Spring 1995	30%***	17%[NS]	2.1	1.3-3.5
Fall 1995	17%	19%	0.8	0.5-1.4

[a] 95% confidence interval for odds ratio.

[b] Logistic regression analysis, adjusted for age, gender, atopy and skin prick test positivity.

*** p<0.001, ** p<0.01 vs. emergency visits or antibiotic courses during fall.

CONCLUSIONS

Our results show that skin test positivity to molds is rare in children at school age. This is true in pupils with asthmatic symptoms, and also in the pupils from the school with moisture or mold problems. Despite this, reactivity to molds seems to be associated with the occurrence of asthma or asthma-like symptoms. In addition, respiratory infections seem to be associated with mold exposure. Asthma and asthma-like symptoms are common in children from moisture or mold problem schools.

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GLUCAN EXPOSURE AND AIRWAYS INFLAMMATION AMONG HOUSEHOLD WASTE COLLECTORS IN TWO MUNICIPALITIES IN SWEDEN

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ABSTRACT

Objective: To study effects of household waste handling two studies were performed. A first study included 17 workers collecting unsorted household waste, eight workers collecting organic/non organic separated waste and 24 controls. The second study included 15 workers collecting organic/non organic separated waste and 27 controls.

Methods: Measurements of airborne endotoxin and (1→3)-β-D-glucan were made in their working environments. Examinations consisted of a questionnaire for symptoms, spirometry, measurements of airway responsiveness, and blood and sputum sampling for determination of differential cell counts, eosinophilic cationic protein (ECP), myeloperoxidase (MPO) and cytokines (study 2).

Results: The waste collectors reported more respiratory and general symptoms as compared with controls. In the first study, the number of blood lymphocytes and monocytes were higher among waste collectors. The effects were associated with higher amounts of airborne (1→3)-β-D-glucan. In the second study, the number of blood lymphocytes was lower and the number of monocytes higher among waste collectors. The exposure levels were lower in this study as compared with the first.

Conclusion: The results suggest that exposure to household waste dust may cause an activation of inflammatory cells.

KEY WORDS:

Household waste, waste collectors, airways inflammation, (1→3)-β-D-glucan, endotoxin, organic dust

INTRODUCTION

Collection of household waste, separation, and composting generate organic dusts which may contain high amounts of endotoxin, bacteria and fungi [Heida et al. 1995]. Respiratory and general symptoms have been reported among workers handling waste [Sigsgaard et al. 1994; Ivens et al. 1997]. In the Netherlands, work related airways inflammation in compost workers was recently reported [Douwes et al. 1997]. Two studies were undertaken to assess the expo-

sure to airborne (1→3)-β-D-glucan and endotoxin and the presence of airways inflammation among household waste collectors.

MATERIAL AND METHODS

In the first study, the organic waste and the non organic waste were sorted separately in plastic bins. The study group comprised all 8 waste collectors collecting compostable waste (full-time) and 17 waste collectors collecting traditional unsorted waste. As controls, 24 persons were recruited from the municipal administration. In the second study, the organic waste was sorted separately in paper bags placed in well ventilated metal bins. The study group comprised all 15 waste collectors collecting compostable waste, at least one day/week. As controls, 27 persons were recruited from a wholesale dealer. All participants in the two studies were males and there were no subjects with physician-diagnosed asthma.

Exposure:

Measurements were made of the amounts of airborne (1→3)-β-D-glucan and endotoxin using personal samplers among waste collectors, and stationary samplers at the work sites of controls. Measurements were made on randomly selected waste collectors. The filters were analyzed for (1→3)-β-D-glucan and endotoxin using specific Limulus lysates [Tamura et al. 1994].

Spirometry and airway responsiveness:

Spirometry was performed using standard techniques. Airway responsiveness was assessed using the methacholine challenge test according to Yan et al. [1983] with some modifications. The methacholine was administered in increasing doses up to a total amount of 1.2 mg. The results were expressed as the group average decrease in FEV_1 after the highest dose of methacholine given.

Questionnaire:

The subjects were interviewed using a questionnaire for organic dust exposure [Rylander et al. 1990].

Induced sputum:

Sputum induction was performed according to Pin et al. [1992] with some modifications [Thorn and Rylander 1998]. ECP (study 1 and 2) and different cytokines (study 2) were determined in the sputum supernatants. The cell pellets were used for differential cell counts.

Inflammatory markers, cytokines (2) and atopy:

ECP was assayed by a FEIA technique and expressed as μg/l. MPO was assayed (in serum only) by a RIA technique and expressed as μg/l. The concentration of specific serum IgE antibodies against ten airborne allergens was assayed by a FEIA technique (Phadiatop test). The results were expressed as positive (atopic) or negative (non atopic). The cytokines, TNFα, IL-5, IL-8 and IL-10 were assayed in sputum samples (study 2) by a enzyme-linked immunosorbent assay.

TREATMENT OF DATA:

The differences between effect variables were analyzed using the Student's t-test and non parametric tests (chi^2 and Mann-Whitney test). Differences were considered statistically significant at $p<0.05$. The studies were approved by the Ethics Committee of the Faculty of Medicine in Gothenburg.

RESULTS

EXPOSURE:

Most of the endotoxin values in the two studies (n=33) were below the suggested guidelines for airways inflammation (10ng/m^3) [Rylander 1997]. At one site with compostable waste higher values were found (40.3 and 12.9 ng/m^3). In study 1, the (1→3)-β-D-glucan levels were highest among those collecting compostable waste (19.1, SD=8.4 ng/m^3, n=7 vs 1.1, SD=2.3 ng/m^3, n=6 work sites among controls). In study 2, higher (1→3)-β-D-glucan values were found at 3 work sites out of 11.

SYMPTOMS:

In study 1, there was a higher proportion of waste collectors reporting lower gastrointestinal symptoms, congested nose, and unusual tiredness as compared with controls. Among workers collecting compostable waste, the differences were statistically significant for lower gastrointestinal symptoms (38% vs 4%, p=0.04) and on borderline for unusual tiredness (25% vs 0%, p=0.056) as compared with controls. In study 2, there was a significantly higher proportion of waste collectors reporting throat irritation, skin rashes, heaviness in the head, joint pains, and flu-like symptoms as compared with controls.

SPIROMETRY AND AIRWAY RESPONSIVENESS:

In study 1, FEV1 in percentage of predicted values was higher among waste collectors as compared with controls (104% vs 97%, p=0.04), but in study 2, there was no difference. There was no difference in the methacholine-induced decrease in FEV1 between waste collectors and controls in the two studies.

INFLAMMATORY MARKERS IN BLOOD:

In study 1, the numbers of lymphocytes and monocytes in blood were significantly higher among waste collectors as compared with controls (3.5, n=25 vs 2.5 *10^9/l, n=24, $p<0.05$ and 0.5, n=25 vs 0.3 *10^9/l, n=24, $p<0.05$, respectively). In study 2, the number of lymphocytes was lower and the number of monocytes higher in blood among waste collectors as compared with controls (1.8, n=15 vs 2.5 *10^9/l, n=27, $p<0.05$ and 0.45, n=15 vs 0.29 *10^9/l, n=27, $p<0.01$, respectively). In both studies, the amounts of MPO and ECP in serum were lower among waste collectors as compared with controls (study 1; MPO 225, SD=45 µg/l, n=25 vs 265, SD=93 µg/l, n=24, p=0.06 and ECP 2.8, SD=1.1 µg/l, n=25 vs 4.7, SD=3.4, n=24, $p<0.05$ and in study 2; MPO 196, SD=89 µg/l, n=15 vs 263, SD=91 µg/l, n=27, $p<0.05$ and ECP 5.0, SD=2.4 µg/l, n=15 vs 7.4, SD=4.0 µg/l, n=27, $p<0.05$).

INFLAMMATORY MARKERS AND CYTOKINES IN INDUCED SPUTUM:

In study 1, the amount of ECP in induced sputum was higher among those collecting compostable waste, and especially among non atopics, as compared with controls (269, SD=164 μg/l, n=7 vs 190, SD=171 μg/l, n=18, NS). In study 2, the amounts of ECP, TNFα and IL-8 in induced sputum were slightly higher among waste collectors as compared with controls (186, SD=110 μg/l vs 166, SD=157, NS, 27.1, SD=22.1 ng/l vs 18.4, SD=18.2, NS and 9.4, SD=5.2 μg/l vs 7.4, SD=5.2, NS, respectively).

The results were largely similar among non-smokers and non-atopics (data not shown).

DISCUSSION

A larger proportion of waste collectors reported general and respiratory symptoms as compared with controls. A higher prevalence of gastrointestinal symptoms among waste collectors as compared with controls was recently reported in another study [Ivens et al. 1997]. An increased prevalence of respiratory symptoms has also been reported in different kinds of organic dusts environments [Carvalheiro et al. 1995; Rylander et al. 1999]. The numbers of monocytes in blood were higher among waste collectors as compared with controls in both studies. Similar results from investigations in other organic dust environments are lacking. The increased number of monocytes in blood could be due to increased stimulation of the immune system. Taken together, the results suggest an inflammatory response among waste collectors. The changes may be a consequence of the waste exposure and of work exposure to (1→3)-β-D-glucan. An influence of the endotoxin exposure cannot be excluded, as experience from animal inhalation experiments suggests that endotoxin activates macrophages and acts synergistically with (1→3)-β-D-glucan [Di Luzio 1985; Fogelmark and Rylander 1994].

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MOISTURE OBSERVATIONS AND HEALTH

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ABSTRACT

Two tenements, one with 252 and the other with 144 separate apartments were studied for their moisture problems and the symptoms of occupants. There were signs of moisture in 97% of the apartments of the index building and in 59% of the reference building. Among adults, the prevalence of nearly all respiratory symptoms was higher in the index building. The prevalence of nocturnal shortness of breath was more than twice as high in the index building than in the reference building. There was a statistically significant difference in adult's visits to a physician because of the respiratory symptoms between the two buildings. When the groups that were defined by the severity of moisture problems in the apartments was considered as an exposure variable, in hoarseness of voice, in nocturnal cough and in nocturnal shortness of breath ORs were high and showed a tendency of dose-response relationship. These results support the conclusion that good maintenance of buildings is necessary in order to prevent moisture problems and corresponding health consequences.

INTRODUCTION

Dampness and mold growth in dwellings have been associated with a number of respiratory health effects (Dales et al. 1991, Jaakkola et al. 1993). The health effects may be caused by the emissions from microbial growth in the structures due to excess moisture. Since excess moisture in structures is the main factor which permits growth of these micro-organisms, observations of dampness and moisture can be used as a proxy of indoor microbial problems and subsequent exposure to microbial emissions (Brunekreef, 1992).

This study was designed to study differences in moisture problems between two tenements. In addition to this, health effects among people living in these two tenements were examined. One of the tenements was assessed as an index tenement and the other as a reference tenement. Also, the aim of the study was to compare health effects between groups that were defined according to the severity of the moisture damages.

MATERIAL AND METHODS

Both the index and reference tenement are located in Tampere in Finland. Tampere is located in the south-west part of Finland. Tampere has little less than 200 000 inhabitants. The index tenement was assessed as index tenement because of the visual moisture and mold observations in the apartments. In addition to this, the tenants of the index tenement suspected that these moisture and mold problems are causing severe health consequences to the people living in the apartments. The reference tenement was selected from the same city with the help of the city landlord. Criteria for the reference tenement was that it should be approximately of the same size and age and the socio-economic structure of the tenants should be the same as in the index tenement. The selected buildings were carefully walked through and all the visual signs of moisture and mold recorded. In the index tenement (built in 1960's) there were 252 and in the reference tenement (built in 1950's) there were 144 separate apartments. An experienced civil engineer made a walk through in 226 apartments of the index building and in 87 apartments of the reference building. The tenants were interviewed according to prewritten questionnaires. The civil engineer made the field study using questionnaires and check lists. Questionnaires on health were sent to the occupants before the visit of the engineer. There were 287 adults from index building from 170 apartments and 102 adults from the reference building from 79 apartments who returned the questionnaire. There were 81 questionnaires returned from the index building from 67 apartments and 23 from the reference building from 21 apartments for children under 16 years old. The field work took place between October, 1995 and January, 1996. Prevalence of symptoms in the index tenement was compared with the same figures of the reference tenement. After that the data from both the tenements were combined for the analysis of prevalence of symptoms between groups that were defined by the severity of moisture problems in the apartments. Potential confounders for the health effects were examined. Odds ratios were calculated as well as 95% confidence intervals for the point estimates of the exposure variables. The differences between the population living in the index building and the population in the reference building were adjusted together with the confounders of the health effects.

RESULTS

There were signs of moisture in 97% of the apartments of the index building and in 59% of the reference building. Among adults, the prevalence of nearly all respiratory symptoms was higher in the index building (table I). The prevalence of nocturnal shortness of breath was twice as high in the index building than in the reference building. There was a statistically significant difference in adult's visits to a physician because of the respiratory symptoms between the two buildings (data not shown). The risk for weakened sense of smell was significantly increased. The prevalence of doctor diagnosed asthma in the index tenement was 8%. The number of asthmatic adults (21) was only a little smaller than the number of adults with allergic rhinitis (25).

TABLE I. Number of tenants having respiratory symptoms and adjusted* Odds Ratios (OR) for adults.

Symptom	Index tenement n (%)	Reference tenement n (%)	OR	95% CI
Dry cough	89 (34)	24 (25)	1.48	0.82-2.68
Cough with phlegm	111 (42)	35 (39)	0.99	0.56-1.75
Hoarseness of voice	26 (11)	6 (7)	2.22	0.71-6.96
Sore throat	33 (14)	9 (10)	1.24	0.52-2.97
Runny nose	72 (29)	19 (22)	1.38	0.72-2.68
Nocturnal cough	60 (23)	15 (18)	1.52	0.75-3.10
Nocturnal shortness of breath	30 (14)	6 (7)	2.22	0.75-6.57
Nasal bleeding	9 (4)	3 (4)	1.19	0.26-5.43
Weakened sense of smell	25 (11)	3 (4)	4.23	1.15-15.5
Eye irritation	103 (40)	48 (50)	0.57	0.33-0.98

*Adjusted for age, sex, smoking, allergy and atopy in family.

In the second phase of the analysis the exposure groups were defined by the severity of moisture problems in the apartments and were classified in three categories. When this was considered as an exposure variable then the ORs were high (table II) in hoarseness of voice, in nocturnal cough and in nocturnal shortness of breath.

TABLE II. Multivariate model* for the prevalence of hoarseness of voice, nocturnal cough and nocturnal shortness of breath.

Variable	OR	95% lower CI	95% upper CI
Hoarseness of voice			
damage severity 1	1.00	ref.	
damage severity 2	4.80	0.51	45.3
damage severity 3	3.80	0.45	31.8
Nocturnal cough			
damage severity 1	1	ref.	
damage severity 2	6.44	0.71	58.0
damage severity 3	12.5	1.58	99.0
Nocturnal shortness of breath			
damage severity 1	1	ref.	
damage severity 2	6.30	0.60	66.5
damage severity 3	7.51	0.83	67.7

*Adjusted for age, sex, smoking, allergy and atopy in family.

DISCUSSION

The main reason for the problems in the index tenement may be lack of maintenance of the buildings. In the reference tenement kitchens and bathrooms had been repaired in the late 80's and in the early 90's, which explains the lower amount of moisture damages in the reference building. Allergic rhinitis is normally three to four times more common than asthma in Finnish adult population. Since the prevalence of asthma was higher and the prevalence of allergic

rhinitis was lower than in the average, it is possible that some of the asthma cases are due to the exposure to moisture damages in the index tenement. Nocturnal cough and shortness of breath are considered early signs of asthma. According to other studies, living in an apartment with moisture damage is associated to the two times higher risk of getting asthma compared with the normal situation (Waegemaekers et al. 1989, Dales et al. 1991, Andrae et al. 1988). Weakened sense of smell was asked the tenants with the questionnaires. Subjectively impaired sense of smell may be due to recurrent or chronic respiratory infections, blocked nose and/or potentially toxigenic microbes due to moisture damages in constructions.

CONCLUSIONS

No serious health effects were associated with exposure to moisture. However, the prevalence of respiratory symptoms and infections was higher in the index building than in the reference building. In combining the whole study material, three symptoms showed tendency to dose-response relationship. These results support the conclusion that good maintenance of buildings is necessary in order to prevent moisture problems and corresponding health consequences.

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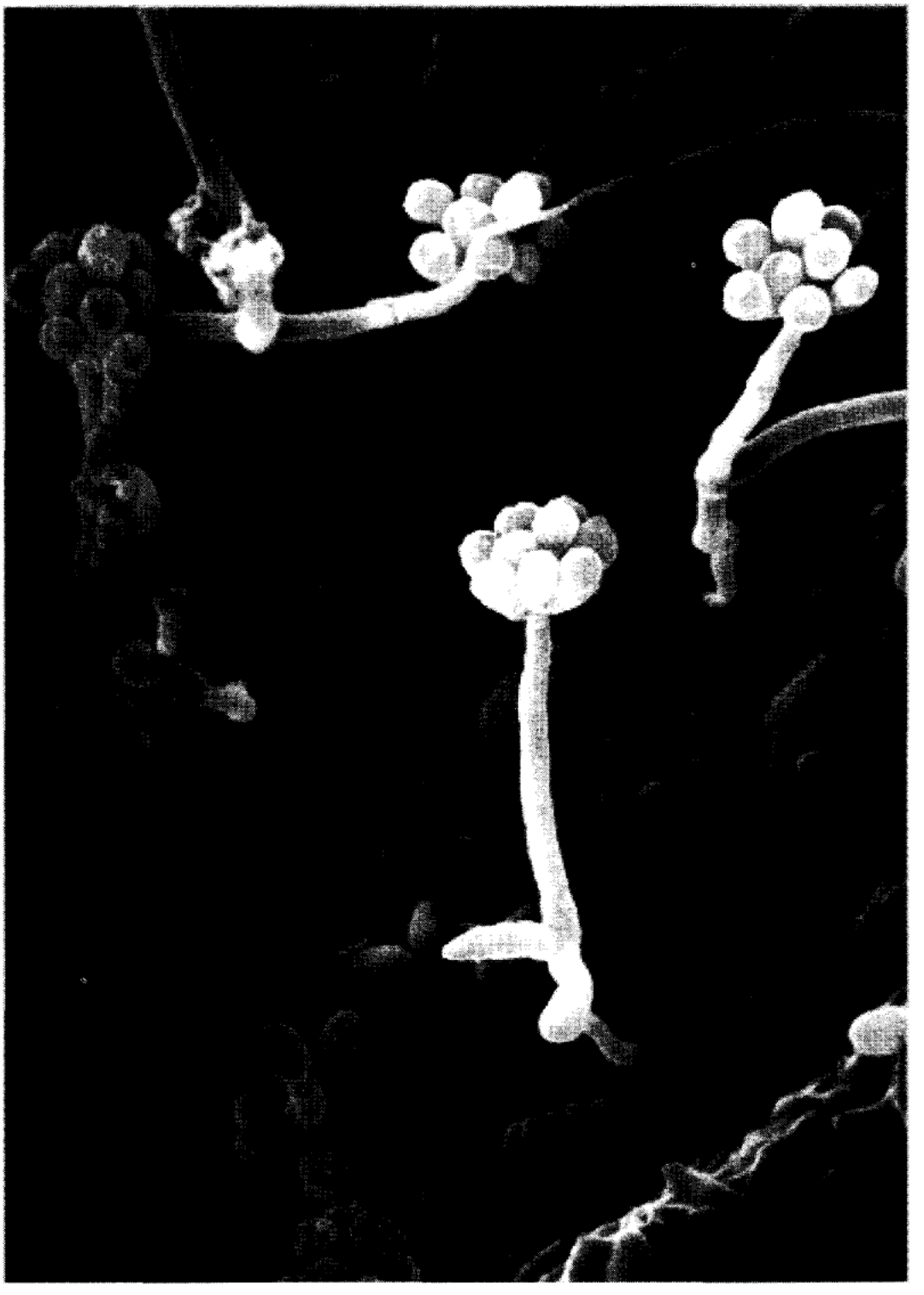
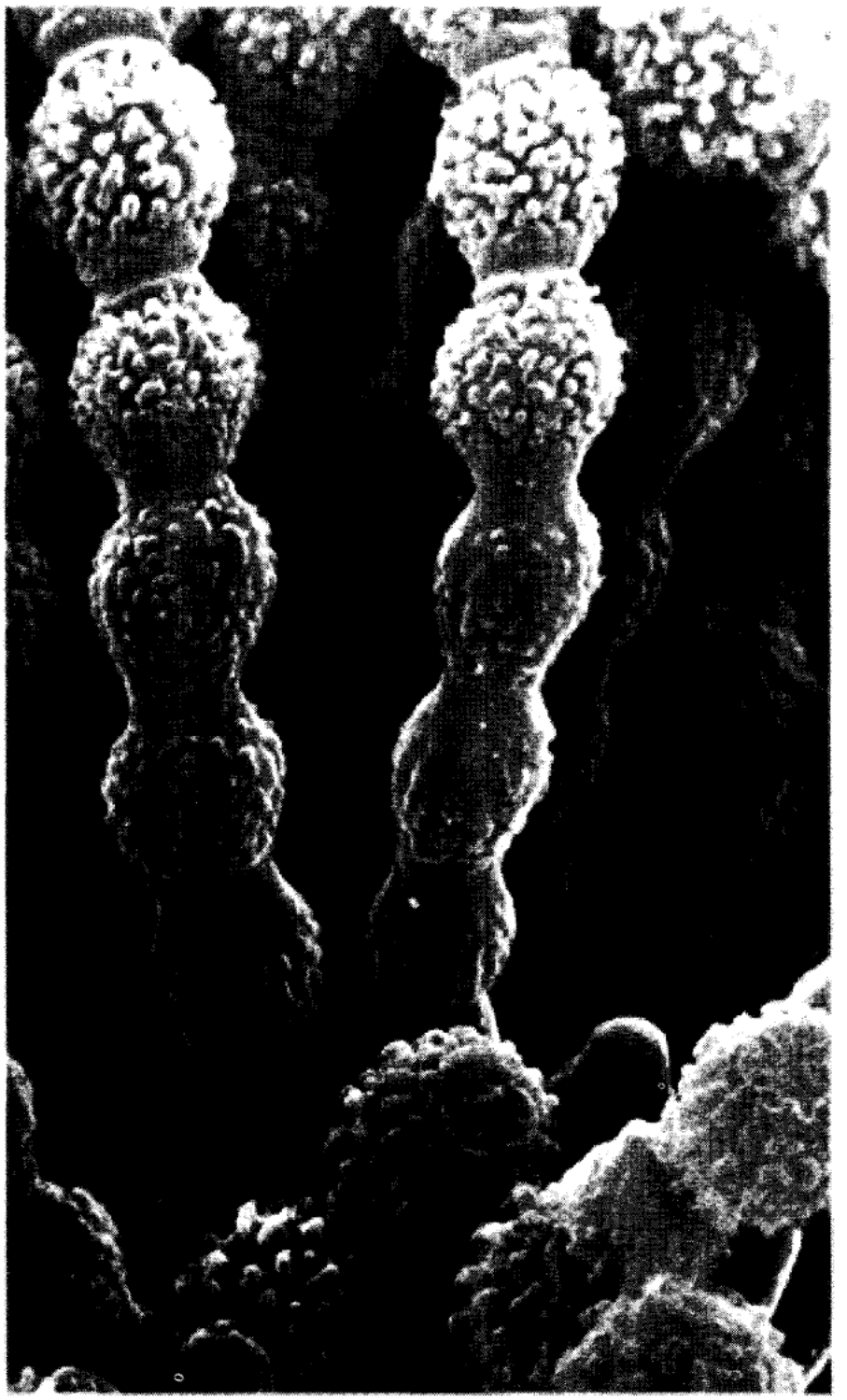


Stachybotrys chartarum, EM 7,000 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands.



Aspergillus, EM 10,600 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands.

Chapter 2

Exposure Assessment Analytical Methods

MYCOTOXIN CYTOTOXICITY SCREENING OF FIELD SAMPLES

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ABSTRACT

Several hydrophil fungi produce very potent chemical metabolites (mycotoxins) that may pose a particular health risk in indoor settings. However, these properties have not been routinely assessed in similar field investigations, due to a lack of readily available screening test methods. A total of 166 molded samples of building materials (gypsum boards, wallpaper, ceiling tiles, fiberglass and other insulating materials, carpets, dust and air filters) from 44 case investigations conducted during the time period from 1993 to 1998 in various areas of the United States were tested with the MTT-cytotoxicity screening assay for the presence of cytotoxic mycotoxins and compared with controls. A subset of samples was analyzed by an enzyme-immune assay (EIA) for occurrence of macrocyclic trichothecenes produced by strains of *Stachybotrys chartarum*. In addition, presence of fungi were examined by culture methods and scanning electron microscopy. Different levels of cytotoxicity were detected in particular of field samples of gypsum board or wall paper visibly contaminated with fungal growth. Samples with highly cytotoxic effects reacted positive in the MTT-bioassay at concentrations of less than 5 mg of sample aliquot per ml of cell culture medium and indicated the presence of cytotoxic contaminants. Twenty-three field samples (52%) of all case investigations were positive for cytotoxicity compared with controls. Most frequently detected fungi in toxic samples were *Stachybotrys chartarum* (85%), *Trichoderma sp.* (31%) and *Chaetomium sp.* (38%) compared to non-cytotoxic samples with a proportion of 38%, 10% and 5%, respectively. These results indicated the presence of non-toxigenic strains or alternatively the non-production of cytotoxic mycotoxins by toxigenic strains on these particular samples. The level of toxicity obtained by the MTT-bioassay highly correlated with the amount of macrocyclic trichothecenes detected by use of the EIA in the samples contaminated with or without *Stachybotrys chartarum*. Toxicity detected in samples not contaminated with *Stachybotrys chartarum* indicates the presence of cytotoxic mycotoxins produced by other fungi. In conclusion, the results support the usefulness of the MTT cytotoxicity assay as an important diagnostic tool for the assessment of potential mycotoxin exposure inside buildings with fungal contamination.

INTRODUCTION

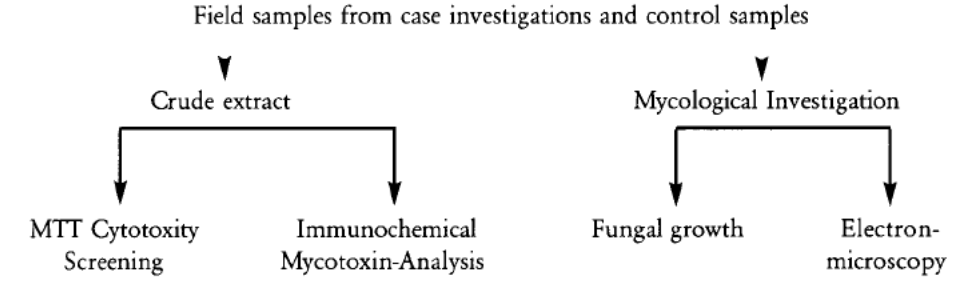
The presence of indoor fungi such as *Stachybotrys chartarum* on water-damaged building materials has been recognized as an important risk factor in exposures to toxic fungal metabolites and associated adverse health reactions (Sorenson, 1990; Hendry & Cole, 1993; Auger et al. 1994, Johanning et al. 1996; Hintikka & Nikulin, 1998). In the indoor environment numerous fungal species belonging to genera such as *Stachybotrys*, *Aspergillus*, *Penicillium*, *Trichoderma*, *Chaetomium* and *Fusarium* could be expected. Concerning the mycoflora of water-damaged building materials, similarities with the food and feed borne mycoflora could be noted (Samson 1998). In general, the formation of mycotoxins on a given substrate is dependent on the genotype of the fungal isolate and a variety of external factors, i.e., composition of the substrate and physical-chemical factors such as the water activity, temperature, pH-value and oxygen. In fungal samples very often the presence of a variety of mycotoxins has to be expected, because many of the above-mentioned fungal species are potent toxin producers and often coexist with each other. In naturally contaminated field samples the extent of mycoflora does not necessarily correlate with the mycotoxin concentration. Isolation of fungi, subsequent cultivation under optimal conditions on artificial media and subsequent chemical analysis of toxin production may not be truly representative of specific indoor environmental situations. Such indirect analyses of field samples may be more of a scientific interest. Furthermore, there is no multi-mycotoxin chemical analysis method available and because of that it is understandable that direct analysis of samples for one or perhaps some mycotoxins with known physical-chemical or immunochemical methods can provide only limited information on the qualitative, i.e. toxicological property of samples in question. Therefore, hygienic exposure assessment needs additional diagnostic tools which improve the direct analyses of field samples and health hazard evaluations.

The objective of this study was to assess the utility of a bioassay in the cytotoxicity analysis of fungal samples, which could be used as a diagnostic tool in the exposure assessment.

MATERIAL AND METHODS

An overview of the testing protocol is shown in figure I. Crude extracts from field samples were analyzed in the cytotoxicity assay and for immunochemical detection of mycotoxins. In addition, presence of fungi on samples was investigated by culture methods and electron microscopy.

FIGURE I: Overview of analytical method assessing fungal samples and corresponding controls.



MATERIALS

A total of 166 samples of building material collected in water damaged homes of occupants who reported significant health problems in time and place related to indoor exposure, were sent to the laboratory for cytotoxicity testing (Table I). The bulk samples were collected during 44 case investigations conducted in various regions of the United States during the time period of 1993 and 1998.

TABLE I: Results of the MTT- cytotoxicity testing of 166 samples from water damaged buildings using the SK target cell line. The bulk samples consisted of 31 different type of building materials and other items. Samples were collected during 44 case investigations carried out in the United States between 1993 and 1998.

Sample material (from n case investigations)	**No. tested***	**No. toxic**	**Non-toxic range of appropriate control samples**
gypsum boards (13)	34	24	> 125 - 500 mg/ml
wall papers (10)	25	20	> 250 mg/ml (stain could interfere)
ceiling tiles (4)	13	7	> 500 mg/ml insulating
insulation materials (fiberglass)	12	9	> 125 - 500 mg/ml (fiberglass could interfere)
air filter - paper (11)	20	4	> 62,5 mg/ml
carpet (5)	5	4	> 125 mg/ml
plaster (2)	4	2	> 125 mg/ml
wood samples (6)	7	3	> 250 mg/ml
miscellaneous samples (7)	16	5	> 250 - 500 mg/ml

* without control samples

MTT-CYTOTOXICITY TESTING

The usefulness of the colorimetric tetrazolium MTT cleavage test for the cytotoxic evaluation of a series of mycotoxin standards, fungal cultures and contaminated samples of different origin has been previously reported (Gareis, 1993; Hanelt et al, 1994; Gareis 1995; Langseth et al, 1997). The principle of this bioassay is based on the transformation of the yellow salt MTT by viable, living cells (via mitochondrial dehydrogenases) to purple formazans. Swine kidney monolayer cells (SK), derived from an adherent cell line with an epitheloid morphology, were used as target cells. These target cells have a proven sensitivity for a wide range of mycotoxins and have been already used in assaying crude extracts from various samples (Hanelt et al. 1994; Gareis 1993).

The target cells were grown to dense monolayers in microtiter plates and then exposed to crude sample extracts. Eight wells of the 96-well tissue culture plate (row 1) remained empty and served as blanks. Row 2 of the plates contained target cells with no exposure to sample extracts and served as cell control. After decanting the cell culture medium (MEM, Minimum Essential Medium with Earl's Salts supplemented with 200 IU penicillin/ml, 200 µg streptomycin/ml and 5% fetal calf serum) from the microtiter plates, 100 µl of the complete medium containing 1.7 % ethanol and 0.3 % DMSO (v/v) were added to each well.

Five to 10 g aliquots of the samples were crushed by cutting and extracted with 150 ml methanol/chloroform (2:1,v/v) for 30 minutes. The extracts were filtered, dried under evaporation and re-dissolved in 5 ml acetone/methanol (2:1, v/v). The organic layers were concentrated to dryness, and dissolved in MEM containing 1.7 % ethanol and 0.3 % DMSO (v/v). On separate plates serial log 2 dilutions of the sample solutions were prepared and 100 µl of each dilution were transferred in duplicates to the cell culture plate. Final concentrations of the crude extract of the samples tested ranged from 3.8 to 500 mg/ml of cell culture medium. All plates were incubated for 20 hours at 37ªC in a humidified atmosphere with 5 % CO2. A volume of 20 µl each of the MTT stock solution was then added to the wells and plates incubated for another 4 hours. Supernatants were then removed using a multichannel micro pipette and 100 µl DMSO was added to each well in order to dissolve the dark formazan crystals. The optical density of each well was measured spectrophotometrically with an ELISA-Reader at a wavelength of 540 nm using row 1 as the blank. Mean extinction values and standard deviations of each sample concentration were compared with those of the corresponding control and expressed as % cleavage activity in comparison to cell controls (100%).

The statistical significance of the test results was calculated by the students t-test at p=0.01. The minimum concentrations of the test reagents to cause toxic effects (cytotoxic endpoints) were determined on the basis of the statistically determined values of 80% cleavage activity.

Mycological examination

Presence of fungal contamination of the samples was investigated by culture and scanning electron-microscopy using standardized methods. Briefly, fungal growth was studied by cultivating small pieces of the sample material on malt extract agar plates for 3 to 5 days at 25ªC. Samples were suspended and transferred by inoculation on filter paper and cultivated up to 14 days at 25ªC to culture *Stachybotrys chartarum*. For electron microscopy, small pieces of samples were dried, fixed with conductive carbon cement and sputtered with gold.

Immunochemical detection of macrocyclic trichothecenes

A subset of crude extracts used for the cytotoxicity assays were analyzed by an enzyme-immune method for detection of macrocyclic trichothecenes and is described in detail by Dietrich et al. (1998).

RESULTS AND DISCUSSION

The field samples submitted for cytotoxicity assay analysis consisted of different materials (building materials) and composition (paper, textile, leather, man-made-mineral fibers, etc.). Crude extract preparations from these various materials may be able to develop a general toxic effect on the SK target cell line, depending on the concentration and not being contaminated with cytotoxic mycotoxins. In order to interpret the cytotoxicological data of a given test sample, it was necessary to test appropriate control samples in order to determine the concentrations at which the target cells could be challenged without showing toxic effects caused only by the matrices (growth substrates). If target cells are used which are too sensitive to matrix

effects, only low sample concentrations could be tested. This may limit the data interpretation. This may even make it impossible to check crude sample extracts by use of a cell bioassay.

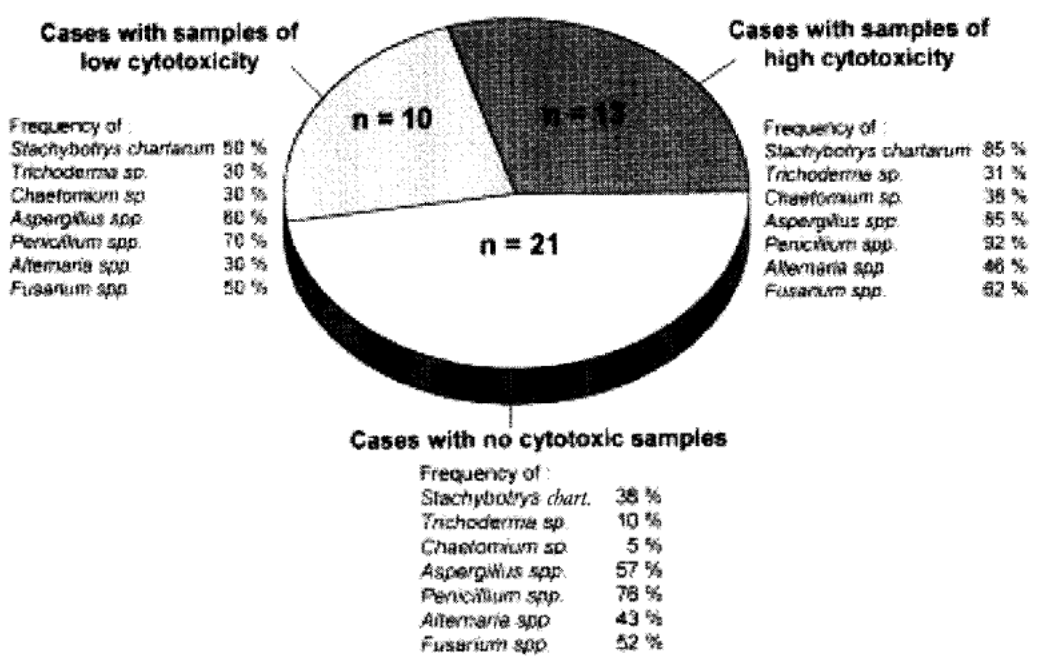
The nontoxic concentrations of crude extracts of sample materials from the case investigations were found to be in a range of 62,5 to > 500 mg of sample aliquot/ml of cell culture medium (Table I). This confirms the appropriateness of the SK cells as target cell-line for the MTT bioassay.

Similar ranges of concentration were also found in previous studies with crude extracts from food and feed. However, it was found that stains from painted wallpapers and also some fiberglass materials could interfere with the test, because of its own high cytotoxicity, possibly because of a high content of heavy metals. To correctly interpret the assay data, it is very important to test appropriate controls as well. Suitable controls are materials (i.e., samples collected from the problem building) which are of the same make-up (matrix) but lack visible evidence of fungal growth.

The findings of the MTT-bioassay tests of a total of 166 samples from 44 case-investigations are summarized in Table I. The majority of the gypsum boards, wall papers, ceiling tiles and insulating materials from these investigations were found to be cytotoxic compared to controls.

In 10 case investigations low sample cytotoxicity was found, in 13 cases high sample toxicity was found (Figure II). No cytotoxicity was detected in samples from the remaining 21 cases. Results of the mycological examinations carried out by culture and electron microscopy showed a higher frequency of *Stachybotrys chartarum* (up to 85% positives), *Trichoderma* sp. (31%) and *Chaetomium* sp. (38%) in cytotoxic samples as compared to samples with no cytotoxic activ-

FIGURE II: Results of cytotoxicity testing and mycological analyses of samples from 44 case- investigations in the United States from 1994 to 1998.



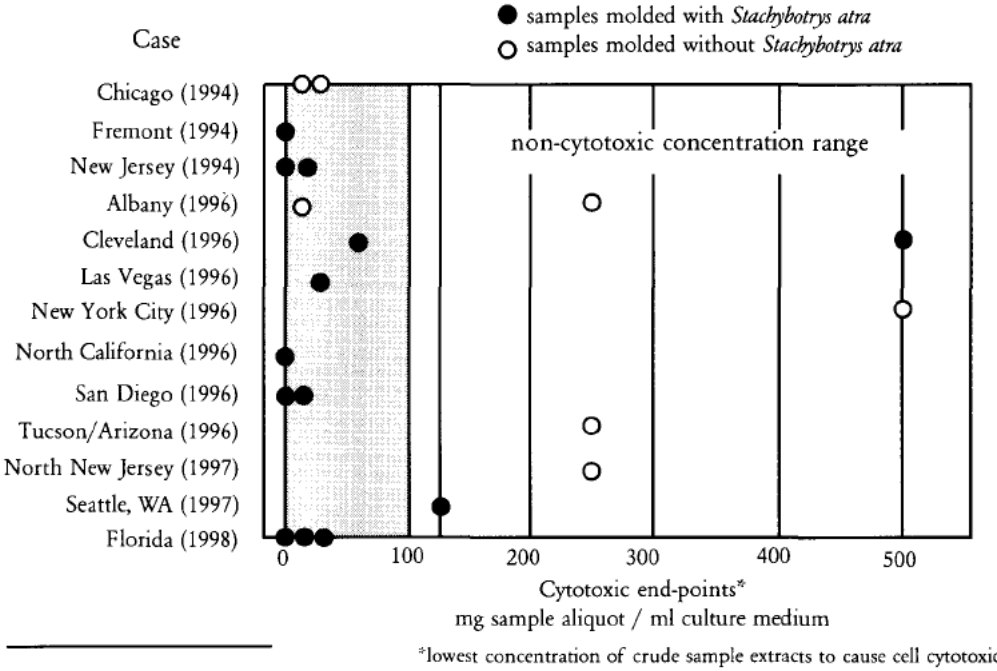
ities. These results indicate that obviously cytotoxic metabolites from these particular fungi may have been present in the samples and may play an important role to cause the toxic effects measured in the MTT-assay.

Strains of *Stachybotrys chartarum* are known to produce the macrocyclic trichothecenes satratoxins which belong to the fungal metabolites with the highest cytotoxicity (Hanelt et al. 1994). The results of the bioassay also show that in molded field samples other mycotoxins than satratoxins should be expected. *Trichoderma viride* is known to produce beside trichothecenes also gliotoxin which also is highly cytotoxic for the SK target cell line used in the MTT-assay.

However, the results also demonstrate that obviously in molded samples from 21 case investigations no cytotoxic contaminants were detected, although isolates of known mycotoxin producers have been identified. This indicates the inability of either toxigenic strains to produce toxins on the particular substrate or that the strains belong to non-toxigenic (or non-producing) isolates.

Frequently paper coated gypsum boards or fiberglass insulation becomes contaminated with fungal growth as a result of water accumulation or moisture problems in buildings. Samples of gypsum boards and wall papers have been analyzed in this study from together 13 case investigations from different geographical regions of the United States. The results of the cytotoxicity tests of these samples molded with or without *Stachybotrys chartarum* are summarized in figure 3. Many of the samples were found to be highly cytotoxic in very low concentrations.

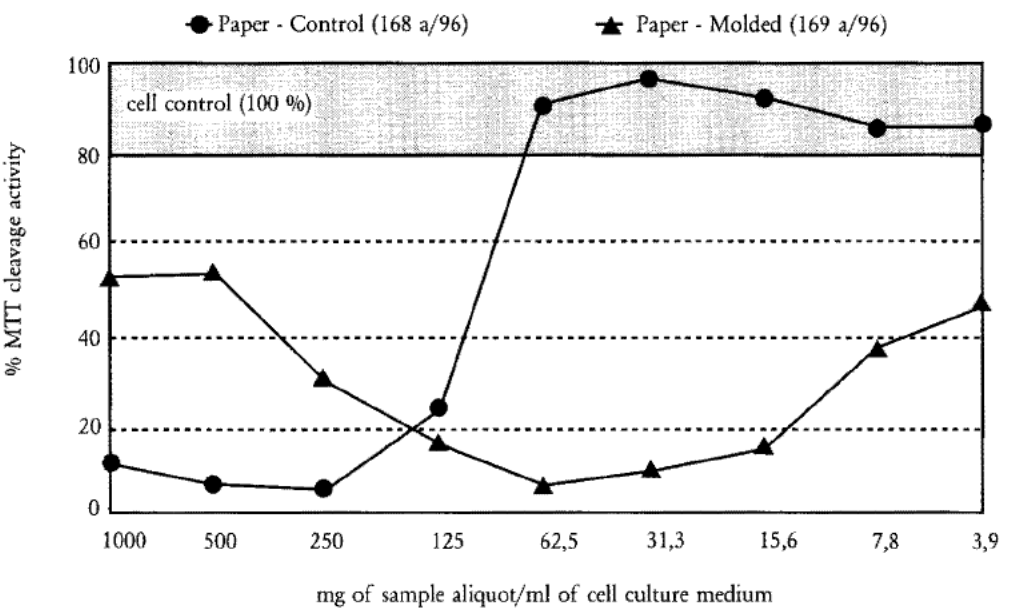
FIGURE III: Results of cytotoxicity testing of molded samples of gypsum boards from 13 case investigations. The non-toxic concentration range for crude extracts of gypsum boards/dry wall papers (i.e. the concentrations of non-molded controls causing toxicity by matrix effects) using the SK target cell line is > 125 mg of sample aliquot per ml of cell culture medium.



However, not all of these samples were contaminated with *Stachybotrys chartarum*. That indicates the presence of cytotoxic fungal metabolites other than satratoxin. One sample molded with *Stachybotrys chartarum* that was not toxic was observed. It may be concluded from these findings that mycological investigation alone may not be sufficient in assessing the health risk caused by mycotoxins.

The figure IV shows the comparison of a visibly molded dry wall paper originating from a case investigation of a water damaged school building in Southern California, USA with a control (dry wall paper). The crude extracts of the field sample were found to be cytotoxic at concentrations as low as 3.9 mg of sample aliquot per ml. The corresponding control reacted positive

FIGURE IV: MTT-cytotoxicity testing of crude extracts of drywall paper from a water damaged school in Southern California, USA, during a case investigation in 1996. Cleavage activities < 80% as compared to non exposed cell control are caused by cytotoxic contaminants.



only at concentrations of 125 mg/ml. The predominate fungi in the visibly molded samples were *Stachybotrys chartarum* and *Trichoderma sp.* compared with the controls (Table. II).

TABLE II: Results of mycological examination of gypsum boards from a water damaged school in Southern California, USA collected during a case investigation in 1996.

Sample	Lab.no.	Germ count	Fungi
Drywall paper (control)	168 a / 96	+ + +	*Fusarium semitectum**
			*Fusarium culmorum**
		+	*Alternaria sp.*
		+	*Cladosporium sp.*
Drywall paper (visibly moldy)	169 a / 96	+ + + + +	*Stachybotrys chartarum*
		+ + +	*Alternaria sp.*
		+ +	*Trichoderma sp.*
		+	*Cladosporium sp.*
			Penicillium sp.
Gypsum (control)	168 b / 96	+ +	*Fusarium semitectum**
		+	*Alternaria sp.*
		+	*Cladosporium sp.*
Gypsum (visibly moldy)	169 b / 96	+ + + +	*Stachybotrys chartarum*
		+ +	*Trichoderma sp.*
		+	*Alternaria sp.*
		+	*Fusarium sp.*
		+	*Penicillium sp.*

+-++++ low to massive

* Determination by Nirenberg, Berlin, Germany

In a case investigation in the North-East region of the USA, various samples including air handling unit filters from a private residence with a history of recurrent water damage (rain storms) and widespread fungal growth was analyzed for cytotoxicity (Figure V, Table III). Crude extracts were also analyzed by the enzyme immune assay, because *Stachybotrys chartarum* was found to be the predominating fungus in these samples. The cell culture assay of the air filter did clearly demonstrate an increased toxicity as compared to the control (Figure V). Indoor bulk samples collected in the house were partially heavily molded with *Stachybotrys chartarum*, confirmed by culture and/or scanning electron microscopy. Compared to controls, these samples were found to be cytotoxic to various degrees. A very good agreement between the EIA results and the MTT-cytotoxicity test could be observed for these samples. Analyses by EIA confirmed the occurrence of macrocyclic trichothecenes in a range of 0.8 to 282.5 ng Roridin A equivalents per g of samples. No toxins were detected in control samples. The excellent correlation between cytotoxicity assay and EIA could be expected for those samples which are contaminated with toxic strains of *Stachybotrys chartarum*. In Figure VI and Table IV examples of this agreement are given. The four samples of dry wall paper, contaminated to various degrees with different moulds but all with *Stachybotrys chartarum*, were from a first class hotel in Florida and collected during a case investigation in 1998. Cytotoxic activities varied between these samples and the cytotoxic end points were in the range of 15,6 to <1,9 mg / ml (Figure VI). High mycotoxin levels (1.124 and 1.164 ng Roridin A equivalents) were detected in the EIA analyses in the most cytotoxic samples. Lower levels (317 and 612 ng/g) were measured in two samples with lower cytotoxic activities.

FIGURE V: MTT-cytotoxicity testing of crude extracts of air filters (control and field sample) from a problem building in the North-East of the USA, during a case investigation in 1996-1997. Sampling was carried out with a fiberglass high air volume sampler over a period of 8 hours.

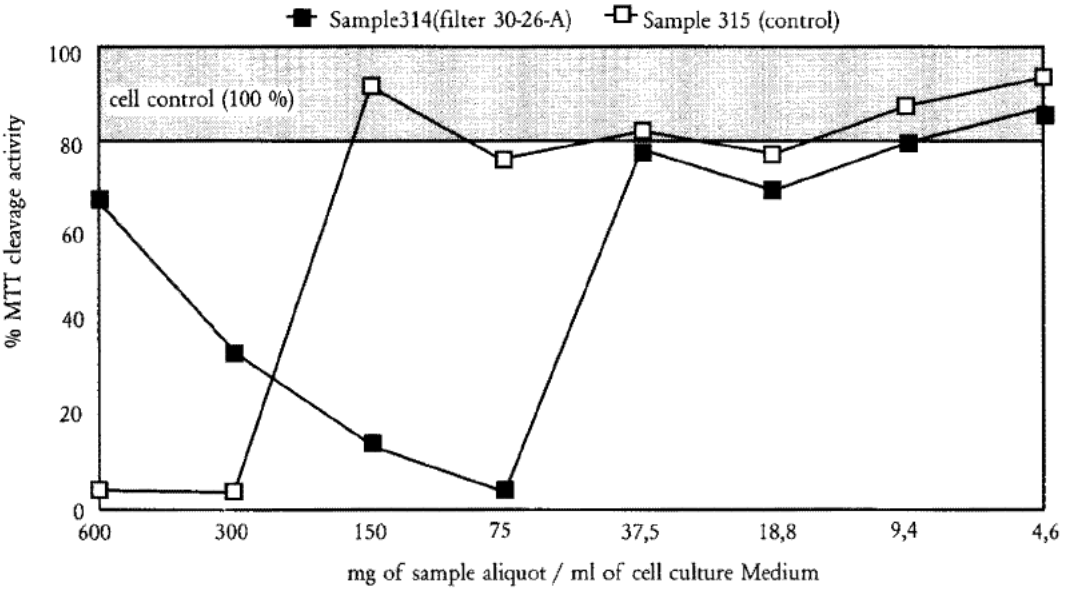


TABLE III: Results of mycological examination, MTT-cytotoxicity screening and immunochemical mycotoxin analyses of indoor bulk samples from a water-damaged house in North-East, USA, collected during case investigations in 1996-1997.

No.	Sample	Location / Type	Visible moulds[1]	Mycoflora	Cytotoxicity[2]	Macrocyclic trichothecene mycotoxins[3] (ng Roridin A equivalents /g of sample)
1	Fiberglass	control	(+)	*Asp./Pen.*	negative	negative
2		ceiling kid's room	+ + +	*St. chartarum/Pen.*	+	3.1
3		ceiling kid's room	+ + +	*St. chartarum*	-	0.8
4		bedroom / kid	+ + +	*St. chartarum/Pen.*	+	1.4
5		fire place (control)	-	-	negative	negative
6		fire place)	+	*Pen.*	+ +	21
7	Drywall /	control kid's room	-	-	negative	negative
8	paper	fireplace	+	*St. chartarum/Asp./Alt.*	+ + +	27.6
9		ceiling office room	+	*St.chartarum/Asp.*	+	7.3
10	Fiberglass / wall paper	office room	+ + +	*St.chartarum/Asp.*	+ + +	282.5

[1] -: negative, (+): low, + - ++++: moderate - heavy *Asp.*: *Aspergillus* sp., *Pen.*: *Penicillium* sp., *St.*: *Stachybotrys*

[2] crude extracts of original samples: - negative, + - ++++: low - high toxicity

[3] Enzyme-immune assay of crude extracts (identical with [2])

FIGURE VI: MTT-cytotoxicity testing of four samples of molded drywall papers originating from a water damaged first class hotel in Florida, USA, during a case investigation in 1998.

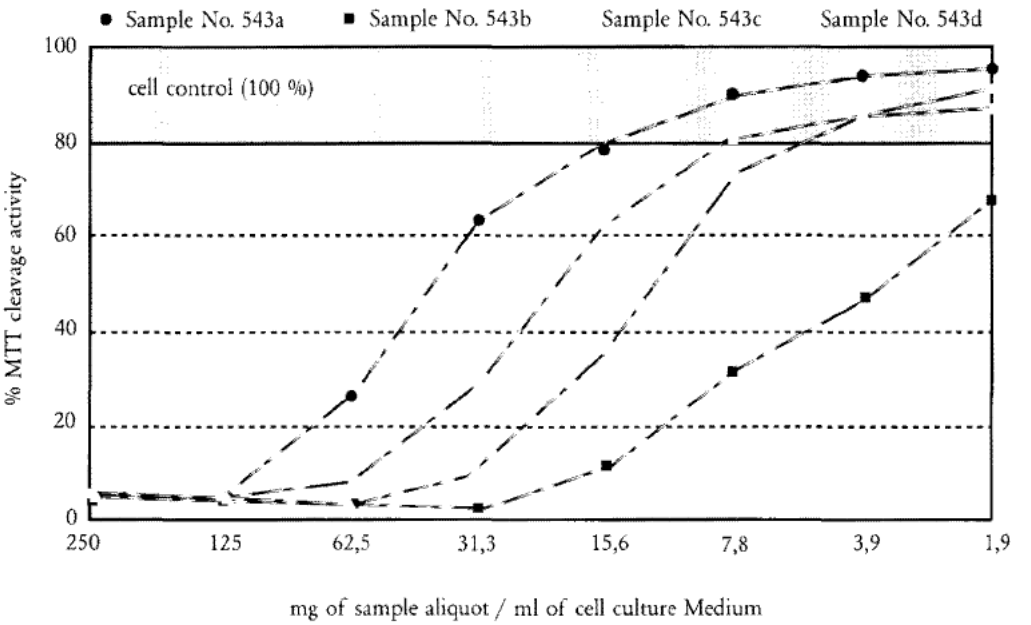


TABLE IV: Results of an analyses of contaminated drywall papers from a water damaged first class hotel in Florida, USA, collected during a case investigation in 1998. Fungal growth was determined by culture methods and scanning electron microscopy. Cytotoxicity of crude sample extracts was measured with the MTT-bioassay. Mycotoxins were analyzed by immunochemical detection (EIA).

Sample no.	Fungal growth	Cytotoxicity	Macrocyclic trichothecene mycotoxins (ng Roridin A equivalents per g of sample)
543 a	*Stachybotrys chartarum* *Aspergillus sp., Aspergillus niger*	+	317
543 b	*Stachybotrys chartarum* *Aspergillus sp., Aspergillus niger,* *Penicillium sp., Syncephalastrum sp.*	+ + + +	1.164
543 c	*Stachybotrys chartarum* *Aspergillus sp., Aspergillus niger,* *Penicillium sp., Syncephalastrum sp.,* *Chaetomium sp., Alternaria sp.*	+ +	612
543 d	*Stachybotrys chartarum Aspergillus sp.,* *Aspergillus niger,Penicillium sp., Syncephalastrum sp.,* *Chaetomium sp., Alternaria sp.*	+ + +	1.124

CONCLUSION

The utility of a bioassay as a useful diagnostic tool depends on the simplicity and quick turn around time. Further, the preparation of crude extracts of various sample materials and an objective interpretation of the results should be possible. The aim of this bioassay was not to study in vitro toxicology but to focus on the screening of qualitative properties of samples. The purpose of the test is to find an answer to the question, are cytotoxic compounds present or not? Further, it should improve and facilitate the detection of exposure to cytotoxic mycotoxins.

The MTT test appears to be a reliable diagnostic method for direct screening of field samples for the presence of cytotoxic compounds, based on the practical experiences with this bioassay during the last five years of case investigations. The SK target cell line has been confirmed to be sensitive for a wide range of mycotoxins but also appear sufficiently robust from non fungal matrix effects from the sample materials. Because crude extracts of samples are tested, not only particular mycotoxins but other possibly cytotoxic compounds are detected. Materials not visibly contaminated should be tested as control samples if available in order to facilitate the interpretation of test results. In combination with the immune chemical method a differentiation between toxic and nontoxic contamination of samples with *Stachybotrys chartarum* fungi appears possible. Toxic field samples were frequently contaminated with *Stachybotrys chartarum*, but also with *Trichoderma viride*, and *Chaetomium*. Using the assay described here will provide information on cytotoxic properties which are characteristic for many but not all naturally occurring mycotoxins.

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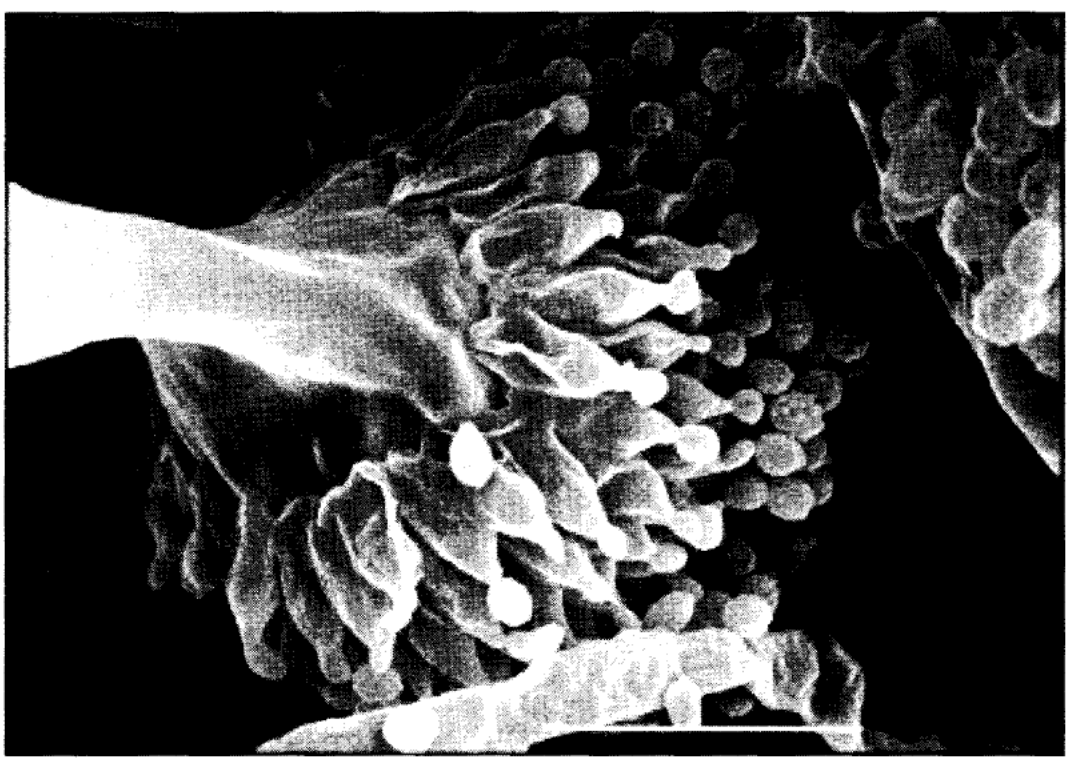

Aspergillus fumigatus, EN 3,300 x, ml 10 mm, dry wall, San Diego 1998,(E. JOHANNING, P.H. SIERCK); M. GAREIS, Institute for Microbiology and Toxicology, Federal Center for Meat Research, D-95326 Kulmbach, Germany.

THE EFFECT OF INHALED SPORES OF MYCOTOXIN PRODUCIN FUNGI ON ANIMALS

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ABSTRACT:

Animals in contact with a moldy environment are exposed to respiratory fungal infection, lung mycosis. Toxic fungal spores in the air create a risk of respiratory effect. Animal experiments have shown that pure mycotoxins administered to the respiratory organs have only a transient or no effect in the lungs. Toxin-containing spores of *Stachybotrys atra* administered to the respiratory organs of mice cause severe hemorrhages and lung inflammation.

KEY WORDS:

mycotoxins, *Stachybotrys*, aerosol exposure, lung mycotoxicosis

INTRODUCTION

Fungal particles, especially spores, are normal components of indoor and outdoor air. Bacteria, virus, different gases, endotoxins and dust are likewise normal contaminants in air (Donham 1991). Toxic fungal metabolites, mycotoxins, in air have been the subject of increasing interest in bioaerosol and indoor air congresses (Hintikka and Nikulin 1998). Aflatoxins (Baxter et al. 1981) and fusarium toxins (Lappalainen et al. 1996) have been found in air samples collected during milling. *Stachybotrys* toxins especially (Jarvis et al. 1995, Etzel et al. 1998) have been suspected as playing a role in environmental problems related to indoor air.

HUMAN EXPOSURE

It is known that fungal toxins often are concentrated in spores (Sorenson 1994). In veterinary medicine mycotoxin publications, cases are reported where people handling straw material that is toxic to animals fall sick as well. Andràssay and colleagues (1980) described a case in Hungary in the late 1970s when 23 people handling straw contaminated by *Stachybotrys* inhaled fungal spores or partly had direct skin contact with the contaminated material. Some persons experienced severe respiratory symptoms such as dyspnoea, throat pain, nosebleeds, and affection of the skin and mucous membranes.

Wu and colleagues (1997) mention a similar case of human exposure. In a small dairy herd, two cows suffered severe dermatitis, respiratory distress, chronic fever and anorexia. The owner of the cattle also suffered skin rashes and respiratory distress. The two cows died and the necropsy revealed hemorrhages in the stomachs and intestine and severe emphysema in the lungs. The oat straw chopped by the cattle owner contained trichothecenes and the affected animals had been placed close to the chopper.

Exposure of animals

A fungal contaminated environment is a risk for animals. Close contact with moldy feed, bedding or moldy indoor/outdoor air exposes animals to direct skin contact or to a risk of inhaling fungal spores and hyphal fragments. Naturally large-scale exposure takes place when animals eating moldy material. Reports on *Stachybotrys* toxicoses in animals mentioned that horses (Schneider et al. 1979, Tantaoui-Elaraki et al. 1994, Le Bars and Le Bars 1996) or sheep (Harrach et al. 1983) were subject to severe effects on the lungs and post mortem showed there to be extensive hemorrhages in the lungs when straw contaminated by *Stachybotrys* had been used as bedding. Moldy bedding is a source of inhalation exposure as well.

MYCOSES OF RESPIRATORY ORGANS ON ANIMALS

Animals, especially farm animals, are often under more serious threat of exposure to mould than people are. Hay, straw and different kinds of bedding materials may contain high quantities of fungal spores. Handling this moldy material may release high concentrations of fungal material into the air. When animals eating forage or grain material, the respiratory organs are exposed to mould spores and hyphal fragments.

Under normal conditions, fungal infections in the respiratory organs of animals are not very common. Humid and warm organic matter like self-heating or fermenting hay, manure, compost, straw and wood shavings are excellent materials for *Aspergillus fumigatus* to breed.

Poultry and confined birds are most susceptible to respiratory mycoses (Korbel et al. 1993). *Aspergillus fumigatus* and *Aspergillus flavus* are the main causative fungi when serious problems occur in poultry hatcheries. Strict sanitary measures are needed to prevent financial losses. Aspergillosis in newly hatched chicks or turkey poults is often acute, with high mortality.

In veterinary medicine, aspergillosis in poultry is a good example of respiratory mycoses where heavy fungal environmental contamination is an important contributory factor of this mycosis.

It is not seldom that animal feed can contain some mycotoxins and at the same time animals are exposed to a host of other infectious agents as well (Niyo et al. 1988). It is clear that animals eating immunosuppressive mycotoxins are more susceptible to microbial infections than animals on a normal diet (Smith et al. 1996).

It is an interesting question in veterinary medicine as to what extent the fungi causing infections in animals are toxin-producing strains, and what is the role of the toxins isolated from infected tissues. Gliotoxin is the most important among the toxins produced by *A. fumigatus*, and this toxin has been detected in fungal affected tissues as well (Bauer et al. 1989). Richard et al. (1996) has isolated gliotoxin in turkey lungs naturally infected with *Aspergillus fumigatus* and the authors emphasis the important point of view that the pathogenic fungus *Aspergillus fumigatus* is able to produce gliotoxin in naturally infected lung tissue. In this way gliotoxin could be involved in the pathogenesis of aspergillosis in turkeys.

Mori et al. (1998) describes a human case of systemic aspergillosis where a toxin producing *Aspergillus flavus* strain had produced aflatoxin in the lung tissue of the patient.

PULMONARY MYCOTOXICOSIS:

A new medical term, "pulmonary mycotoxicosis", was introduced by DA Emmanuel and his group way back in 1975 (Emanuel and Co 1975). He pointed out that the term "mycotoxicosis" involves a toxic reaction due to the oral ingestion of toxins produced by some fungi. The authors described the cases of ten patients who had inhaled massive amounts of fungi that had resulted in an apparent toxic pulmonary reaction called pulmonary mycotoxicosis. The patients had been exposed to extremely moldy silage when cleaning silos.

Several animal experiments have been conducted in order to study the effect of inhaled mycotoxins. Two different methods have been used; either pure mycotoxins in a solvent (Table 1) or mycotoxin containing spores (Table 2.) have been administered to the respiratory organs.

TABLE I: Effect of pure mycotoxins on experimental animals.

Author	Toxin exposure	Animal species	Toxic effect
Ueno 1984	T-2, culture filtrate	mice	death
Marrs,co 1986	T-2, comparison with s.c.exposure	guinea pig	similar toxic effect, no lung toxicity
Creacia, Thurman, co.1987-90	T-2, nose-only	mice, rat, guinea pig	death, inhalation 10-20 times more toxic, no pulmonary injury, general T-2 toxicosis
Pang, co. 1988	T-2, endotracheal tube	swine	changes resemble those of T-2 i.v.
Jakab, co. 1994	aflatoxin	mice, rat	suppresses pulmonary and systemic host defense

TABLE II: The effect of toxin-containing *Stachybotrys atra* spores on mice. Toxic *Stachybotrys atra* strain (strain no 72) produced satratoxins G and H, *stachybotrys* lactones and -lactams. Less toxic/non-toxic strain (strain no 29) produced only minor amounts of *stachybotrys* lactones and -lactams. (Nikulin et al. 1996, 1997).

	Toxin exposure	Effect
Group 1 one intranasal administration/ 3-days experiment/ 1 million spores	toxic strain	death, severe intra-alveolar and interstitial inflammation and hemorrhages in lungs, necrotic changes in lungs
Group 2 one intranasal administration/ 3-days experiment/ 1 million spores	less / non -toxic strain	mild inflammation in lungs
Group 3 six intra-nasal administrations / 3-weeks experiment	toxic strain	severe inflammatory changes and hemorrhages in lung
Group 4 six intranasal administrations / 3-weeks experiment	less / non-toxic strain	mild inflammation in lungs

EXPERIMENTS WITH PURE TOXINS:

Ueno (1984) reported that inhalation of T-2 toxin solution or a culture filtrate of *Fusarium* sporotrichoides containing T-2 toxin caused death in mice.

Marrs and colleagues (1986) compared the inhalation and subcutaneous routes of T-2 toxin in guinea pigs. Their experiments showed that the toxic effect was quantitatively and qualitatively similar using either of the two routes. Histological changes in the target organs were independent of the route used and the toxicity of T-2 by inhalation was not markedly different from the other routes. No specific toxic effects on the lungs were observed.

Several papers on acute inhalation toxicity of T-2 mycotoxin have been published by the group of C.D. Creasia, J.D. Thurman and colleagues. In their first paper Creasia and colleagues (1987) published the results of the inhalation toxicity of T-2 mycotoxin in both young and adult mice. T-2 toxin was dissolved in ethanol and the type of inhalation exposure chamber was for nose-only exposure. Mice exposed to higher concentrations in the experiment died within a few hours. Experiment results showed that the inhalation of T-2 mycotoxin by mice is at least 10 times more toxic than systemic administration and at least 20 times more toxic than dermal administration. Thurman et al. (1988) described the histology of mice exposed to aerosolised T-2 toxin. Creasia and colleagues (1990) studied the inhalation toxicity of T-2 on rats and guinea pigs using the same experimental model used earlier for mice. Their experiment results showed that inhaled T-2 toxin was approximately 20 times more toxic to the rat and at least twice as toxic to the guinea pig than ip-administered toxin. In these experiments, the lesions after respiratory exposure were similar to those after the systemic administration of T-2 toxin. It is worth noting that no gross or histological evidence of acute pulmonary injury was found.

Pang with colleagues (Pang et al. 1988) reported the effects of inhalation of T-2 toxin in swine. A toxin aerosol was administered through an endotracheal tube and the changes resembled those reported in pigs after T-2 toxin by iv-injection. The authors suggest that because of the lipophilic nature of T-2 toxin, it may be rapidly absorbed from the lungs, metabolized and excreted. This could explain the lack of effects on the lungs and the similarities between the effects following inhalation exposure and iv-administration.

Jakab and colleagues (1994) exposed rats and mice to aflatoxins and demonstrated that experimental respiratory exposure of AFB 1 suppressed pulmonary and systemic host defenses.

EXPERIMENTS WITH TOXIC SPORES:

Instead of pure mycotoxins in experiments by Nikulin et al. (1996) spores of *Stachybotrys atra* were used to investigate lung mycotoxicosis. A group of mice received only one intranasal injection of spores of a highly toxic strain of *S. atra* and another group received the same injection of a less toxic strain. The toxic spores contained satratoxins G and H and stachybotrylactones and lactams. The spores containing satratoxins caused severe intra-alveolar, bronchiolar and interstitial inflammation with hemorrhages. The spores without satratoxins induced a milder inflammation. The authors concluded that the toxic compounds of *S. atra* spores are most likely responsible for the severity of the lung injury. In a second experiment, (Nikulin et al. 1997) six intra-nasal injections were administered over three weeks. The mice receiving the higher concentration of toxiç *S. atra* spores developed severe inflammatory changes and hemorrhages in the lungs, whereas no histological changes were detected in the target organs for

trichothene toxicosis. No IgG antibodies were detected in the sera of mice exposed intra-nasally to *Stachybotrys* spores. In these two experiments toxic *Stachybotrys* spores caused hemorrhages and inflammations in the lungs without any effects in the spleen, thymus and intestine.

IN VITRO EXPERIMENTS/CYTOTOXICITY OF MYCOTOXINS IN TISSUE AND CELL CULTURES

Cell culture tests are nowadays used as a tool to test the toxicity of different materials including mycotoxins (Gareis 1994). These tests can be used e.g. to screen samples for mycotoxins or for semi-quantitative assessments of the toxicity of a sample.

The cytotoxic effects of mycotoxins have been studied also by using various lung tissue and lung cell culture techniques (Ball et al. 1995, Zarba et al. 1992, Donnelly et al. 1996, Stewart et al. 1996, Mitchell et al. 1997, Larsson and Tjälve 1996). The results of these experiments can help figure out the possible.significance of fungal material and mycotoxins in the inhaled air.

CONCLUSIONS:

1. Inhaled fungal spores can cause lung infection, toxic effect in the lungs (lung mycotoxicosis) or allergic reaction.
2. Inhaled T-2 toxin has only a transient effect in the lungs and causes changes similar to general mycotoxicosis.
3. Inhaled toxic *S. atra* spores cause inflammatory changes and hemorrhages in the lungs and no changes in the target organs for *stachybotrys* toxicosis.
4. *Aspergillus fumigatus* and *A. flavus* are able to produce toxins in lung tissue.

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TRICHOTHECENES AS A POTENT INDUCER OF APOPTOSIS

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ABSTRACT

Induction of apoptosis by the trichothecenes was examined in promyelotic leukemia cell line HL-60 and mice. An early elevation of intracellular Ca^{++} ion followed by disruption of observed mitochondrial transmembrane potential, induction of caspase-3, and DNA fragmentation was observed in T-2 toxin-treated cells. An induction of apoptotic cell death in the tissues of mice given T-2 toxin was confirmed by electron microscopic analysis. The induction of apoptosis was proposed as a novel cell death mechanism of T-2 toxin as well as macrocyclic trichothecenes.

KEY WORDS:

Trichothecenes, T-2 toxin, verrucarins, satratoxins, HL-60 cells, mice Apoptosis, DNA fragmentation, caspases, Ca^{++} ion, electron microscope

INTRODUCTION

Based on numerous biochemical approach to the mode of action on toxic fungal metabolites, they are grouped into 1) genotoxins (aflatoxins et al.), 2) mitochondrial impairments (luteoskyrin et al.), 3) cytoskeletal modifiers (cytochalasins), 4) protein synthesis inhibitors (trichothecenes, ochratoxins et al.), and 5) an estrogenic hormone (zearalenone), as reviewed (Ueno 1985). Pathologically, most of the mycotoxins are reported to induce necrotic cell death in their target organs.

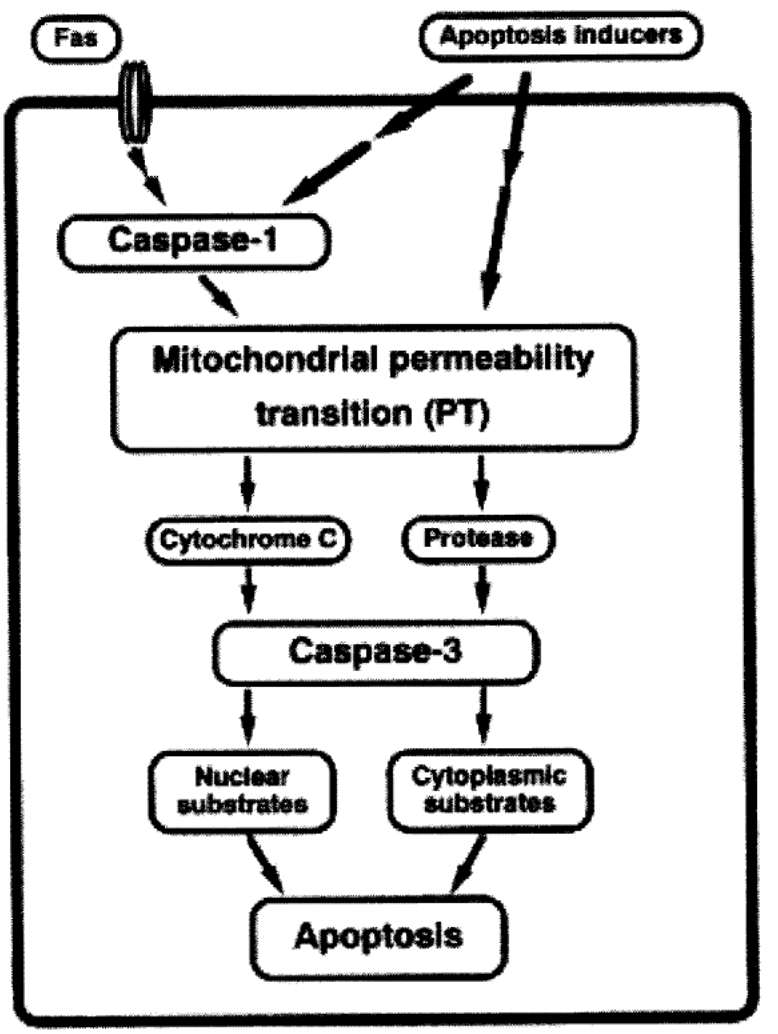
Current concept on cell death proposed an another mechanism, apoptosis (Wyllie et al. 1980). The necrosis is characterized by mitochondrial swelling and degeneration of organella. While, the apoptosis, which plays an important role for development and homeostasis in humans and animals, is a cell death characterized by condensation of cytoplasma and fragmentation of nuclear DNA. This apoptosis is associated with several human diseases including cancer, Alzheimer's disease and acquired immunodeficiency syndrome (AIDS) (Figure I), and induced by several external stimuli such as anticancer drugs, radiation, toxic chemicals, along with an internal factor Fas, as schematically shown in Figure II.

Figure I: Association between apoptosis and human disease.

Diabetes mellitus	AIDS	Acute hepatitis	Alzheimer disease
Viral infection/ autoimmune disorder?	HIV infection	Viral infection	Genetic and/or acquired?
↓	↓	↓	↓
			amyloid ppt
↓	↓	↓	↓
B-cell	T cell	hepatocytes	nervous cell

- Apoptotis is a common cell death mechanism
- Suppression of common apoptotic cell death by chemicals lead to development of novel drugs for therapy

Figure II: Signal transduction in apoptotic cell death.



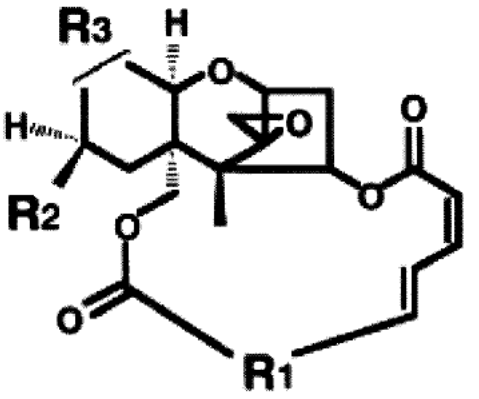
After finding the apoptotic cell death, the mechanisms of cytotoxic chemicals are re-investigated in cellular and animal levels by analyzing DNA fragmentation, electron microscopic changes and expression of apoptosis-related genes and products.

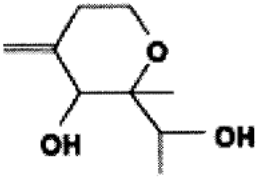
In this communication, we tried to clarify whether the apoptosis is involved in the cell death induced by the cytotoxic fungal toxins. Particularly, the experiments were focussed to the trichothecenes, since T-2 toxin (T-2), nivalenol and deoxnivalenol produced by various genera of plant-pathogenic *Fusarium spp.* contaminate world-widely agricultural commodities resulting in food-borne diseases (Ueno 1986; Tanaka et al. 1988). The macrocyclic trichothecenes such as satratoxins produced by an air-borne fungus *Stachybotrys atra* (Eppley et al. 1973) (Figure III) are suspected as a risk for pulmonary diseases and leukemia (Croft et al. 1986; Hintikka and Nikulin 1998; Johanning 1998). Beside these fungal trichothecenes, a toxic plant Baccharis produces potent cytotoxic macrocyclic trichothecenes, baccarinoids (Kupchan et al. 1977; Jarvis et al. 1980)(Figure III).

Previously, we reported that the trichothecenes exhibit an inhibition of protein synthesis in mammalian cells and tissues, and this biochemical impairment was considered to a reason for their cytotoxic nature (Ueno et al. 1973). The present results indicated that the non-macrocyclic trichothecenes such as T-2 as well as the macrocyclic trichothecenes sush as roridin A, verrucarins and baccarinoids possess a potential for an induction of apoptosis in human promyelotic leukemia cell line HL-60 and rodent peripheral blood lymphocytes in vitro. Detailed experiments with T-2 as a model of the trichothecenes revealed an early elevation of intracellular Ca^{++} ($Ca^{++}i$), followed by a disruption of mitochondrial transmembrane potential ($\delta\psi m$), an activation of caspase-3 and DNA fragmentation. Electron microscopic observation and in situ nick translation technique also confirmed an induction of apoptosis in tissues of mice given the trichothecenes.

A part of the present results was reported as a preliminary form (Okumura et al. 1998a,b)

Figure III: Structure of macrocyclic trichothecenes.



Compound	R1	R2	R3
Verrucarin A	CHOHCHCH3CH2CH2OCO –	H	
Roridin A	CHOHCHCH3CH2CH2OC(OH)(CH3)–	H	
Baccarinoid B4	CHCCH3CHOHCH2OCH– (epoxide O; C(OH)(H)(CH3))	OH	
Baccarinoid B5	CHCCH3CHOHCH2OCH– (epoxide O; C(CH3)(H)(OH))	H	
Baccarinoid B3	CHOHCHCH3CH2CH2OCH– (C(CH3)(H)(OH))	OH	
Satratoxin H		H	

METHODS

Mycotoxins

The most of mycotoxins examined were isolated in our laboratory, except roridin A and verrucarin A were purchased from Sigma. Satratoxin H and baccharinoids were isolated by the co-authors E.-L Hintikka (Finland) and B. Jarvis (US).

Cells and animals

Human promyelotic leukemia cell line HL-60 were cultured in RPMI 1640 medium supplemented with 10 μg/ml of gentamicin and 10% heat-inactivated fetal calf serum, and various concentrations of test chemicals dissolved in ethanol. The final concentration of ethanol was fixed to 0.1%. ICR female mice were *i.p.* given a sub-lethal dose of 5 mg/kg of T-2 toxin or 4.1 mg/kg of nivalenol, and the tissues were processed for electron microscopic observation and *in situ* nick-translation immunostaining method.

DNA fragmentation

HL-60 cells ($5x10^5$ cells) were exposed to the test chemicals for various times. Analysis of DNA fragmentation was carried out as reported (Ueno et al. 1995), briefly as follows. The cells were harvested, washed with 200 μl of buffer A (10 mM Tris-HCl buffer, pH 7.8, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 300 μM phenylmethyl sulfonyl fluoride (PMSF), and lysed in lysis buffer (50 mM Tris-HCl buffer, pH 7.8, 10 mM EDTA, 0.5% sodium N-lauroyl sarcosinate). The lysed cell suspension was treated sequentially for 60 min at 50°C with 200 μg/ml of DNase-free RNase A and 0.5 μg/ml of proteinase K. Electrophoresis of fragmented DNA was carried out in 2.0% agarose gels in TAE buffer (40 mM Tris-acetate buffer, pH 8.1, 2 mM EDTA) containing 0.5 μg/ml ethidium bromide at 100 V for 1 hr. DNA fragmentation pattern was examined in photographs under UV illumination.

Laser cytometric analysis of intracellular Ca^{++}

The cells were loaded with 10 μM of the acetoxymethyl ester of Fluo-3 (Fluo-3 AM) and 1% of Cremophor EL. This Fluo-3 AM is permeable through the cell membrane and hydrolyzed in the cytoplasm into the impermeable form Fluo-3 which selectively chelates with $Ca^{++}i$. After exposed to the toxins for various times, the labeled cells were imaged using an IN-Sight plus-IQTM laser scanning confocal microscope system equipped with an Olympus IMT-2 inverted microscope, as reported (Yoshino et al. 1996).

Caspase activity

HL-60 cells ($1x10^7$ cells) treated with 100 ng/ml of T-2 for various times were washed with ice-cold phosphate buffered saline (PBS), The cell pellets were suspended in 100 μl of extraction buffer (50 mM Pipes-NaOH buffer, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20 μM cytochalasin B, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain, 10 μg/ml chymopain), and the cytosolic extracts were prepared by freezing and thawing 5 times in liquid nitrogen. For estimation of caspase activity, the cytosolic extract diluted with ICE standard buffer (100 mM Hepes-KOH buffer, pH 7.5, 10% sucrose,

0.1% CHAPS, 10 mM DTT, 100 μg/ml ovalbumin) were incubated at 30°C for 30 min with 1 μM of the specific fluorescent substrates, Ac-YVAD-MCA for caspase-1 and Ac-DEVD-MCA for caspase-3. The amounts of 7-amino-4-methyl coumarin (AMC) released from the above fluorescent substrates were estimated by HPLC fluorometry (Okumura et al. 1998c).

Flow cytometry and mitochondrial membrane potential δψm

For estimation of cell viability, the cells treated with the chemicals were washed with PBS and fixed with 500 μl of 70% ethanol for 20 min on ice. Then, the cells were incubated with 100 μg/ml of RNase A for 20 min at 37°C and stained with 25 μg/ml of propidium iodide (PI) for 20 min on ice. For analysis of δψm, the HL-60 cells ($5x10^5$ cells) treated with T-2 were washed twice with ice-cold PBS, stained with 40 nM of a δψm-sensitive dye, DiOC6(3) for 15 min at 37°C. Control cells were simultaneously treated with the protonophore mClCCP. Analysis of incorporated fluorescent substrates was carried out on a FACScan (Becton Dickinson Immunocytometer system) equipped with an argon laser tuned to 488 nm using LYSYS II software. PI and DiOC6(3) incorporated were recorded as FL2 and FL1 respectively (Okumura et al. 1999).

RESULTS AND DISCUSSION

Screening of apoptosis inducers in fungal toxins

Based on the analysis of DNA fragmentation in HL-60 cells, more than 40 fungal metabolites including the mycotoxins and antitumor agents were tested with actinomycin D as a positive control. As reported (Ueno et al. 1995), the trichothecenes (roridin A, T-2, nivalenol, deoxynivalenol), luteoskyrin, ochratoxin A, rubratoxin B, gliotoxin, emestrin, citrinin and 2-hydroxyemodin were positive for this assay. While, no significant DNA fragmentation was observed with cyclochlorotin, phalloidin, fusaric acid and others irrespective of their cytotoxic nature. It means that the cytotoxic mycotoxins could be grouped into two categories, apoptosis-inducer and -non inducer.

Among the inducers, the potential of trichothecene mycotoxins was remarkably high, as summarized in Table I. The baccharinoids, isolated from toxic plants and chemically closed to roridin A and satratoxins, also induced DNA fragmentation (Figure IV).

These results revealed that the macrocyclic trichothecenes tested induced the DNA fragmentation in HL-60 cells at extremely low concentration, although their activity was slightly different dependent on their structure. The non-macrocyclic trichothecenes such as nivalenol, which world-widely contaminated cereals and foods (Tanaka et al. 1988), is also positive but far less potent than the macrocyclics.

The induction of apoptosis was also observed in peripheral blood lymphocytes exposed to nivalenol (Yoshino et al. 1997a) and T-2 (Yoshino et al. 1997b), suggesting that the induction of apoptotic cells is not restricted to established tumor cell lines.

Figure IV: DNA fragmentation by the macrocyclic trichothecenes.

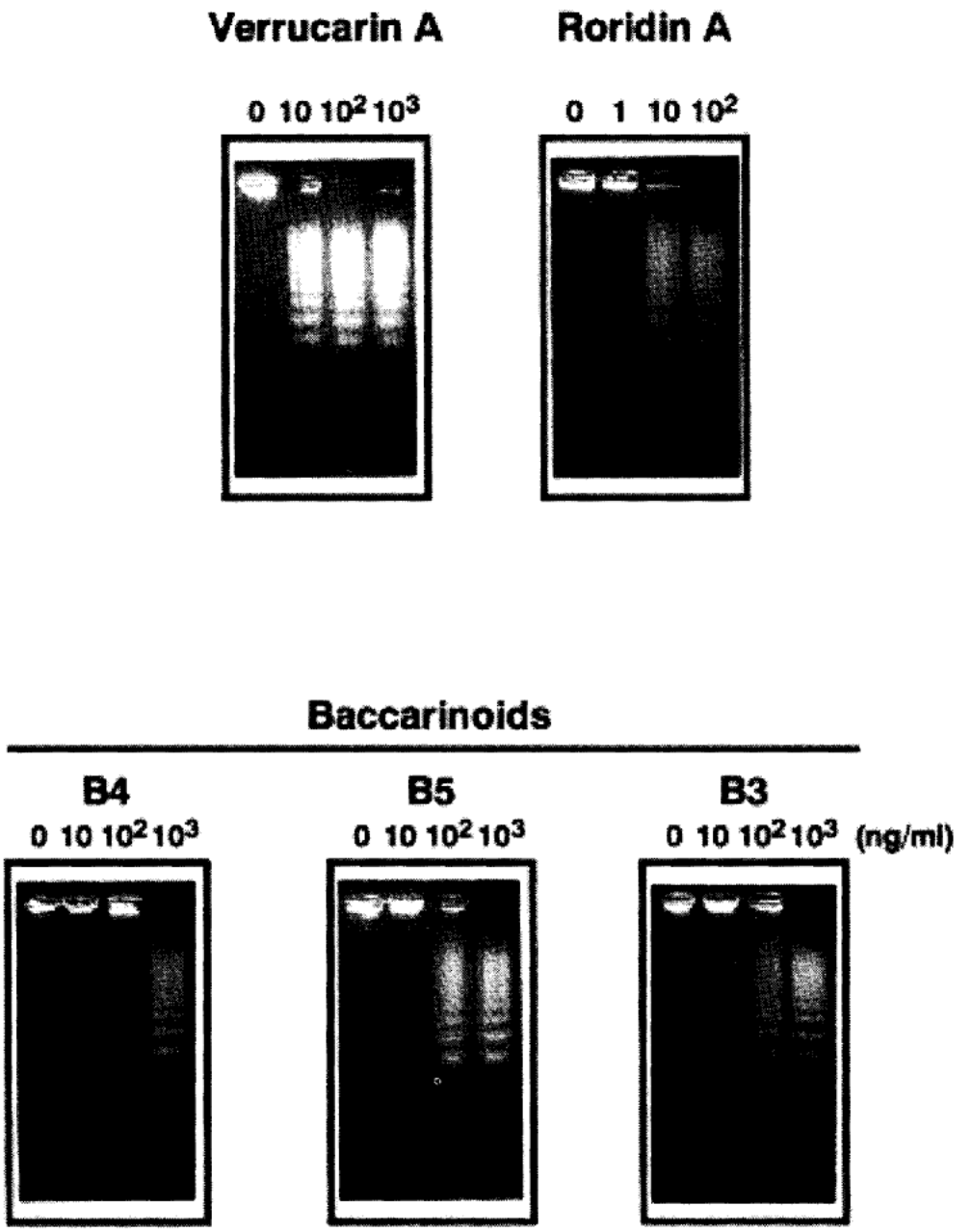


TABLE I: Comparative cytotoxicity and apoptosis induction (μg/ml).

Trichothecenes	Cytotoxicity (IC_{50})	Induction of apoptosis (MEC)
T-2 toxin*	0.004	0.01
Nivalenol*	1.0	0.1
Roridin A*	0.0005	0.001
Roridin A	0.001	0.01
Verrucarin A	0.0008	01
Baccharinoid B4	0.4	0.1
Baccharinoid B5	0.05	0.1
Baccharinoid B3	0.09	0.1
Satratoxin H	0.01	0.01

IC_{50}= 50% inhibition concentration for cell growth
MEC=Minimum effective concentration for apoptosis
*Incubation time; the three marked were for 12 hr and the others for 6 hr.

INDUCTION OF APOPTOSIS IN ANIMALS

In order to demonstrate the induction of apoptosis in whole animals, the cellular architecture of the selected organs of mice given the trichothecenes was examined under electron microscope. In mice given nivalenol, the apoptotic cell death was marked in the thymus (Sugamata et al. 1997). The nuclei became invaginated and pinched off to give fragments, and crescent-shaped spaces (CSS) were found in early stage (Sugamata et al. 1998a). The apoptotic cell death was also observed in peripheral blood lymphocytes of mice given nivalenol (Yoshino et al. 1997a). In the thymus and spleen of mice given 5.0 mg/kg of T-2, a massive cellular destruction characterized by chromatin condensation was evident. In the liver, the cellular changes characterized by apoptotic body and its elimination by phagocytosis by Kupffer cells were observed. In situ nick-translation analysis by TUNEL method revealed DNA fragmentation in the liver, spleen and thymus (Sugamata et al. 1998; Ihara et al. 1998). These findings showed that the trichothecenes induce the apoptosis in cellular- and whole-animal levels. An important morphological finding is that the formation of CSS in the nuclei is an early biomarker for apoptotic cell death in vivo.

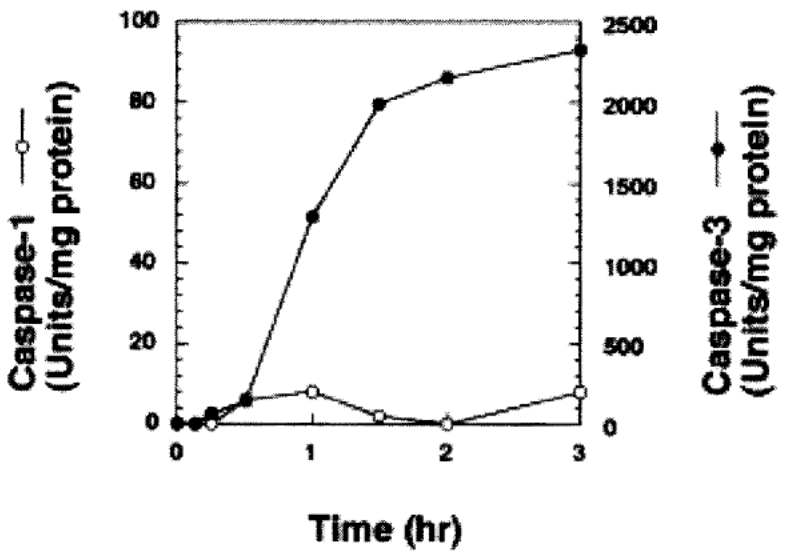
TRANSIENT ELEVATION OF INTRACELLULAR Ca^{++} AS AN EARLY EVENT OF T-2-APOPTOSIS

Since the function of several intracellular enzymes/factors is highly regulated by cytoplasmic ions, particularly of Ca^{++} ion, the level of $Ca^{++}i$ was monitored time-sequentially under confocal microscope. The results revealed $Ca^{++}i$ in the cells exposed to 100 ng/ml of T-2 sharply elevated about six-fold above the control after the incubation only for 4 min, and returned to an initial level within 30 sec thereafter. This transient elevation is an early event in T-2 -treated cells. As well established, several intracellular enzymes are activated by Ca^{++}ion, resulting in an alteration of signal transduction. We presumed that T-2 attacks firstly intracellular Ca^{++}storages such as endoplasmic reticulum (ER) to release Ca^{++}ion bound, and the resulted elevation of $Ca^{++}i$ switches "on" the machinery for apoptosis, then the activated proteases and endonucleases induce cleavage of nuclear proteins and DNA. The elevated free $Ca^{++}i$ may be immediately trapped by Ca^{++} scavenger, mitochondria.

Activation of caspase-3

As mentioned in Introduction and Figure II, caspases, a group of cystein proteases, is key enzymes for apoptotic cell death. When the cells were exposed to various concentrations of T-2, the level of caspase-1 was not changed, while that of capase-3 was elevated (Figure V). This marked elevation was observed within 1.5 hr prior to the DNA fragmentation of cells even when exposed to a low concentration (6 ng/ml) of T-2. Furthermore, an evidence that Ac-DEVD-CHO, an inhibitor of caspase-3, blocked T-2-induced apoptosis supported the present finding that T-2 induces the apoptosis through a common signal pathway of apoptosis. No activation of caspase-1 suggested that Fas-induced apoptosis is not contributed for the induction of apoptosis by T-2 (Okumura et al. 1999).

Figure V: Activation of caspase-3.by T-2.



Disruption of potential of mitochondrial transmembrane potential

Disruption of $\delta\psi m$ during apoptosis and necrosis is a critical step in cell death (Kroemer et al. 1997). T-2 caused this disruption 0.5-1 hr prior to the cell death. Electron microscopic observation revealed a condensation of the mitochondria of the cells exposed to T-2. Generally, morphological preservation of mitochondria is a characteristic in apoptotic cell death. In this respect, the apoptosis observed by T-2 is different from other cases in regards to mitochondrial morphology (Okumura et al. 1999).

SUMMARY

The present findings presented a strong evidence that the trichothecene mycotoxins are a potent inducer of apoptosis *in vitro* and *in vivo*, and this apoptotic cell death is mediated by an

early elevation of $Ca^{++}i$ followed by the disruption of mitochondrial transmembrane potential and the activation of caspase-3, resulting in DNA fragmentation. Since the air-borne macrocyclic trichothecenes such as verrucarins possess a potential for the induction of apoptosis at an extremely low dose, their role as a risk factor for indoor human diseases such as lung hemorrhage, inflammation, immunodepression and leukemia is suspected.

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ANALYSIS FOR *STACHYBOTRYS* TOXINS

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ABSTRACT:

Analytical conditions have been developed for the detection and quantitation of several classes of mycotoxins produced by the toxigenic mold, *Stachybotrys atra (S. chartarum).* Fungal cultures or environmental samples are extracted with methanol-chloroform and the crude extracts passed through a PEI silica cleanup column. The fractions from this cleanup column are analyzed by reversed phase chromatography (C-18, acetonitrile-water-formic acid) coupled to a diode array detector. Three major classes of mycotoxins are produced by *S. atra:* trichothecenes, phenylspirodrimanes, and, a new class of diterpenoids, the atranones. Thirty-eight isolates of *S. atra* were assayed for their mycotoxin production, and the *S. atra* isolates fell into two distinct classes: those that produce trichothecenes (12/38) and those that produce atranones (22/38).

KEY WORDS:

trichothecenes, immunosuppressants, HPLC-diode array detection, mycotoxins, *Stachybotrys, Memnoniella*, diterpenes

INTRODUCTION

Historically, fungal toxins (mycotoxins) have been of concern in human and animal health in agricultural settings (Betina 1989). These toxins are best known as a risk to the health of humans and animals through ingestion of contaminated food and feed, although workers handling contaminated grains and other contaminated agricultural material are susceptible to other modes of mycotoxin exposure. Exposure through inhalation is the next most likely significant mode of exposure to toxigenic molds.

In the past twenty years, there is increasing recognition that an important factor in the health of people in indoor environments is the dampness of the buildings in which they live and work (Miller 1995). Furthermore, it is now appreciated that the principal non-pathogenic biologics responsible for the health problems in such buildings are fungi rather than bacteria or viruses (Jarvis 1995). One factor associated with certain fungi are small molecular weight toxins (mycotoxins) produced by these fungi (Miller 1995). Traditionally, mycotoxins are held to be important in human and animal health because of their production by toxigenic fungi associated food and feed (Betina 1989). However, mycotoxins tend to concentrate in fungal spores (Larsen et al. 1994; Sorenson et al. 1987), and thus present a potential hazard to those inhaling airborne spores.

Stachybotrys atra (S. chartarum) is one of the more hazardous of the toxigenic fungi found in wet buildings. *S. atra* produces the very potent cytotoxic macrocyclic trichothecenes, along with a variety of immunosuppressants (Jarvis et al. 1995; Kaneto et al. 1994; Sakamoto et al. 1994) and endothelin receptor antagonists (Ogawa et al. 1995; Roggo et al. 1996a; Roggo et al. 1996b). This fungus was held responsible for serious health problems of a family living in a water-damaged home in Chicago (Croft et al. 1986) and has been implicated in several cases of building related illness (Hodgson et al. 1998; Johanning et al. 1993; Johanning et al. 1996). A cluster of cases of acute pulmonary hemorrhage/hemosiderosis was reported in Cleveland, Ohio where over 30 infants from homes that suffered flood damage became sick (nine deaths) with the illness starting in January 1993 (MMWR, December 9, 1994; MMWR, January, 1997). An investigation by CDC in cooperation with several laboratories (including that of BBJ) has concluded that *S. atra* (which was found in nearly all the case homes and at levels considerably above those in control homes) was responsible for this syndrome (Etzel et al. 1998).

MATERIALS AND METHODS

General

Cultures of *S. atra* (Jarvis et al. 1986; Etzel et al. 1998) were grown on rice as described previously (Jarvis et al. 1998). Details of the isolation and characterization of the atranones will be given elsewhere (Hinkley et al. 1999 (3)).

Caution: S. atra cultures may cause serious rashes. Handling and extractions of cultures should be conducted in a chemical fume hood. Since *S. atra* is not a pathogen, one need not autoclave the culture before chemical extraction.

Extraction and Cleanup

Dark black rice culture was transferred to a small coffee grinder and coursely ground. About five grams of ground rice culture was accurately weighed into an Erlenmeyer flask and treated to ultrasound (1 hr) in chloroform-methanol (1:1, 30 mL). The mixture was allowed to stand overnight (4°C), treated to a further ultrasonic agitation (1 hr), then filtered. The extraction was repeated (without overnight standing) and the filtrates combined. Solvent removal by rotory evaporation gave the crude extract as a black gum.

A portion (~50 mg) of the crude extract was accurately weighed and transferred to a vial, and a slurry generated with PEI silica (75 mg) and MeOH/CH_2Cl_2 (1:1, 0.3 mL). The mixture was evaporated to dryness using rotary evaporation to give a free flowing powder. A 3 mL filtration column (JT Baker) was packed with PEI silica (1 g) and pre-washed with hexane (10 mL) (Jarvis et al. 1998). The PEI-extract mixture was transferred to this column, and a small amount of hexane (0.5-1 mL) was used to wash all of the PEI from the vial onto the top of the column. The column loaded with extract was eluded with the following solvents: hexane (15 mL, fraction I), dichloromethane (15 mL, fraction II) and MeOH (15 mL, fraction III). Solvent was removed from fractions II and III using a stream of N_2 or by standing overnight. The residue was quantitatively taken up in MeOH (2 x 1 mL) and filtered (0.45 μm) into vials suitable for HPLC analysis.

HPLC ANALYSIS

High performance liquid chromatography (HPLC) analyses were carried out on a Hewlett Packard 1100 series HPLC instrument equipped with an autosample injection. All samples were filtered through 0.2 μm PTFE filters (Phenomenex, 13 mm) as MeOH solutions and injection volume maintained at 5 μL. Column temperature was regulated at 40°C and separation effected with a guard column (15 x 2 mm hand packed with Rainin C-18, 8 μm) and main column (150 x 2 mm Phenomenex sphereclone C-18, 3 μm).

The solvent system comprised water (A) and acetonitrile (B), each with 0.1% formic acid (88% Fisher). The solvent program was a ramp from 25% to 80% B at time 0 to 30 minutes, then increased to 100% B from 30 to 31 minutes, and maintained at 100% B until 40 minutes. The solvent system was returned to 25% B (over a 2 min period) and the column re-equilibrated for 10 minutes at a flow rate of 0.2 mL min^{-1}.

Detection was effected with a diode array detector, and chromatograms were recorded at 210, 230, 260, 280 and 300 nm. UV/Vis spectra were saved every 0.5 seconds from 200 to 400 nm and data were processed employing the HP software and computer system.

Peak identification was achieved by comparison of UV/Vis spectra for individual peaks in the chromatogram recorded at 260 nm, against library data. A retention time window of ±5 % was permitted, and spectral match of >950 out of a possible 1000 was required for positive identification. Peaks so identified were calibrated against external standard curves of linear correlation 0.999 or better.

TABLE I: Retention times (RT) in minutes upon C-18 HPLC chromatography (see details above) of trichothecenes and atranones produced by *S. atra*.

Trichothecenes	**RT**	**Atranones**	**RT**
Trichoverrol B	10.2	Atrenone A	22.2
Roridin L-2	15.4	Atrenone B	22.3
Satratoxin G	15.6	Atrenone C	24.9
Satratoxin H	16.5	Atrenone D	25.4
Isosatratoxin F	17.5	Atrenone E	27.0
Isosatratoxin G	19.3		
Roridin A	19.5		
Verrucarin A	20.2		
Verrucarin B	23.2		
Epiisororidin E	24.0		
Isororidin E	24.3		
Roridin E	25.1		
Epiroridin E	25.3		
Verrucarin J	26.9		
Roridin H	30.8		

GC-MS PREPARATION AND ANALYSIS

To a portion of crude extract (~20 mg in MeOH) was added an aliquot of internal standard solution (triphenylene, 25 μL, 3.9 mg mL^{-1} solution in CH2Cl2). The mixture was treated to

hydrolysis at room temperature (NaOH/MeOH solution 5% w/v, ~250 µL, 2 hr), after a period of ultrasonic agitation (1 hr). A glass pipette was charged with ion-exchange resin (400 mg, Rexyn 300 (H^+ / OH^-), Fisher), contained by tightly packed cotton wool plugs, and washed with MeOH (1 mL). Once the hydrolysis was complete, the mixture was discharged onto the resin with three washes of MeOH (200 µL each). The column was washed with CH2Cl2 (1 mL), and the eluant was evaporated under a stream of dry nitrogen. Silylation was achieved by reaction in neat bis(trimethylsilyl)trifluroacetamide (BSTFA, Aldrich, 0.1% TMS, 100 µL) in a capped vial at room temperature for one hour. The reaction mixture was diluted with CH2Cl2 (1 mL) and filtered through cotton wool into a vial suitable for GC-MS analysis.

GC-MS analysis was completed on a Finnigan GCQ with column conditions: initial 130°C, ramp to 150°C (over 1 min), ramp to 250°C (over 17.7 min), ramp to 300°C and hold to purge column; carrier gas He at constant velocity of 40 cm s^{-1} on a Resteck column (RTX-5MS, length 30 m, ID 0.25 mm). Injector 220°C, transfer line 200°C, source 280°C. The MS was run with full scan mode from 100-420 AMU. Injection was by an AS9000 autosampler with a split ratio of 25:1 and 1 µL sample size. The internal standard triphenylene had a retention time of 16.5 min and the bis-TMS-verrucarol had a retention time of 15.1 min. The internal standard and verrucarol derivative were readily identified by their fragmentation patterns.

RESULTS AND DISCUSSION

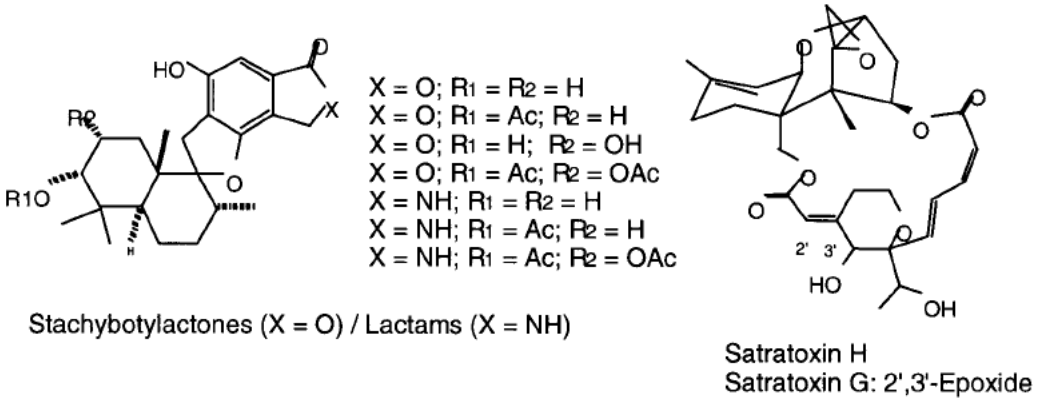
Most of the attention focused on *S. atra* as a health risk to people living and working in *Stachybotrys*-contaminated buildings has been for the potential of *S. atra* to produce the potent trichothecene mycotoxins. Animal intoxications caused by ingestion of *Stachybotrys*-contaminated fed are well documented (Forgacs 1972), Workers handling *Stachybotrys*-contaminated straw (Andrassy et al. 1979), and other material (Dill et al. 1998) also experienced numerous symptoms attributable to trichothecene toxicosis. Furthermore, animal experiments have demonstrated that inhalation is a particularly effective route for toxin administration since the trichothecenes are ca. an order of magnitude more toxic by this route than by ip or iv injection routes (Creasia et al. 1987). Intranasal administration of highly toxigenic spores of *S. atra* to mice resulted in extensively more lung tissue damage compared to that observed when spores of a less toxigenic isolate of *S. atra* were administered (Nikulin 1996; Nikulin 1997). The damage observed correlated with the levels of trichothecenes produced by these molds.

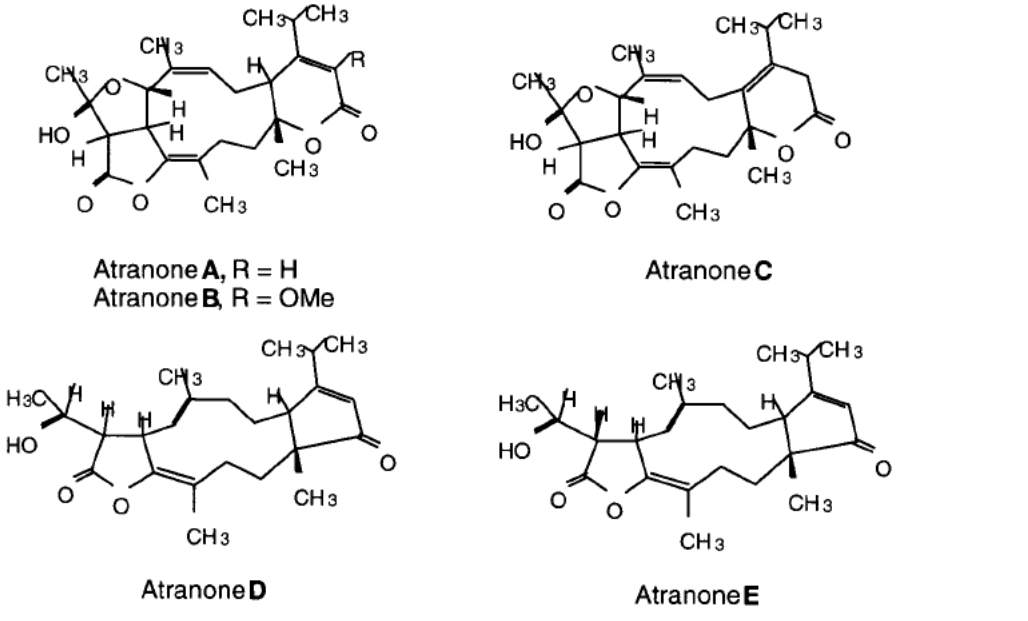
In our recent paper on the analysis of the *S. atra* cultures from the Cleveland study, we reported that 11 of 18 *S. atra* isolates produced macrocyclic trichothecenes and related trichoverroids (Jarvis et al. 1998). The most common trichothecenes found were the satratoxins (isoF, G, iso G, and H) and roridin L-2. We have analyzed an additional 20 *S. atra* isolates from the Cleveland study, five of which produced trichothecenes, ranging in levels from 20 to 360 ppm; satratoxins as well as roridins E and isoE were found in all the trichothecene-containing samples.

As in the earlier study (Jarvis et al. 1998), these latter 20 isolates of *S. atra* all produced varying levels of the immunosuppressant phenylspirodrimanes (stachybotrylactones and lactams). Thus we conclude that these latter compounds are signature compounds of *S. atra*, since we have yet to find an isolate of *S. atra* that does not produce detectable amounts of these com-

pounds (Figure I). One of the isolates from the earlier study also produced a small amount of dechlorogriseofulvins (Jarvis et al. 1998), These compounds are produced in substantial quantity by a related fungus, *Memnoniella echinata*, which was also isolated from a Cleveland home (Jarvis et al. 1996), but none of the latter 20 isolates of *S. atra* appeared to produce any detectible quantities of griseofulvins.

FIGURE I: Structures of *S.atra* Toxins.





In an earlier survey of Eastern European isolates of *S. atra*, we found several isolates which did not appear to produce trichothecenes (Jarvis et al. 1986). A careful examination of a large scale culture of one of these isolates (Debrecen 5142) confirmed the absence of tri-

chothecenes and the presence of the expected spirodrimanes. TLC analysis of the crude extract, following spraying of the plate with vanillin spray and subsequent heating, revealed several distinctive bright orange spots. A series of chromatographies lead to the isolation of C24 compounds of novel structure. Their structures were established by a combination of spectroscopic and X-ray crystallographic data (Hinkley et al. 1998). We have named these compounds atranones (Figure I), and they are a heretofore undescribed class of compounds. The diterpenoid dolabellanes, produced by brown algae and bryophytes (e. g. liverworts) (Asakawa 1995) are the most closely related natural products to the atranones. The atranones have appended to C-6 of the bicyclic dolabellane ring system an additional C4 unit, which has a further connection to the dolabellane ring via a unique enol lactone. Another unusual feature of the dolabellanes is the apparent biosynthetic relationship between atranones **A-C** and atranones **D** and **E**, which differ in the A-ring by an oxygen atom insertion between carbons C-1 and C-14. This transformation appears to be the result of a biosynthetic Baeyer-Villiger reaction, more commonly encountered in bacteria than fungi (Walsh and Chen 1988; Wright et al. 1996). However, examples of this transformation are known in both fungi (Minto & Townsend 1997) and plants (Felico et al. 1986).

A careful examination of all 38 Cleveland *S. atra* isolates has shown that none of the trichothecene-producers yield any atranones, and none of the atranone-producers give any detectable amounts of trichothecenes. This latter point was checked by hydrolyzing crude extracts (NaOH in MeOH), derivatizing any verrucarol formed as the bis-trimethylsilyl ether and analyzing by GC-MS. This is a highly sensitive method, and none of the extracts from the atranone-producing isolates of *S. atra* produced verrucarol. Furthermore, we have gone back and reassayed the 18 isolates of *S. atra* reported earlier (Jarvis et al. 1998) and find that four isolates (JS5807, JS5816, JS5819, and JS6301), which we reported as producing low levels of trichothecenes, in fact produce none of the trichothecenes but instead produce atranones. Thus, the revised numbers for the 38 isolates of *S. atra* from the Cleveland study, with respect to toxin production are as follows: 12 trichothecene producers, 22 atranone producers, and 4 isolates which appear to produce neither the trichothecenes nor the atranones. All extracts were hydrolyzed, and the hydrolyzates derivatized to yield trimethylsilyl ethers. The bis-trimethylsilyl ether of verrucarol was easily detected in the extracts of the 12 trichothecene producers but was completely absent in the remaining 26 extracts. We also have checked carefully for production of griseofulvins by these 38 *S. atra* isolates but find, as reported earlier (Jarvis et al. 1998), only one isolate of *S. atra*, JS5806 produced this class of mycotoxin, then only in trace amounts.

CONCLUSIONS

About a third of *S. atra* isolates produce trichothecenes, and of those, about half are highly toxigenic. The levels of trichothecenes in the producing *S. atra* isolates range from a few ppm to as high as 300 ppm. About 60% of *S. atra* isolates produce atranones, whose levels are on the order of 10^2 - 10^3 ppm. The acute cytotoxicity associated with *S. atra* isolates is due mainly to the trichothecenes, but other natural products produced by this fungus also have biological activities, which may contribute to the overall toxicity of this mold.

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ASSESSING BIOAEROSOLS IN ELEMENTARY SCHOOL CLASSROOMS

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ABSTRACT:

A two year study of elementary school classrooms was begun in 1996. Data collected included: number of occupants and patterns of occupancy, CO_2 levels, temperature and percent relative humidity (%RH), total particulate, and air exchange rates using tracer gas (SF_6). Indoor and outdoor bioaerosols were collected using an Andersen N-6 impactor.

The effect of measured indoor air quality parameters and building characteristics on the airborne concentrations of indoor bacteria and mesophilic fungi were evaluated using multiple linear regression. The variable with the highest predictive value for bacterial concentrations was indoor CO_2 concentration, with the final equation having a R^2 of 0.59. In contrast, outdoor temperature had the highest predictive value for indoor mesophilic fungi with the final equation having a R^2 of 0.61.

Variables commonly measured during an indoor air quality investigation can be used to understand bioaerosol concentrations. High commensal bacterial counts are clearly related to inadequate ventilation of the occupied space. In a similar manner, the use of mechanical or natural ventilation in buildings and the concentration of fungi in the outdoor environment is related to indoor fungal counts. These data would suggest that indoor air quality investigations benefit from an integrated investigative approach.

INTRODUCTION:

In our metropolitan area, in common with other urban areas, the publicity and awareness of mould as a potential marker for poor air quality has led to a proliferation of investigations in buildings which include measurements of bioaerosols. Investigators commonly use numeric guidelines once proposed by the ACGIH bioaerosols committee (Burge et al. 1987), or by Health Canada (Federal-Provincial Advisory Committee, 1995). However, the ACGIH has renounced using numeric guidelines, and has placed the interpretation of bioaerosol measurements back to the expertise of the investigator. Fungal bioaerosols are ubiquitous indoors and

outdoors, and this study intended to examine non-complaint environments in order to elucidate background levels for future comparison.

In total, three rooms in each of 39 schools were studied (n=116). Schools were randomly assigned to a fall (mid September to first hard frost in November), winter (mid January to end of February) or spring (April to mid June) sampling period. Two seasonal cycles were required to complete all the schools. Information collected included building age, building construction, occupancy patterns, furnishings, evidence of previous or current water damage and meteorological conditions. Measurements were taken of indoor carbon dioxide concentration, air exchange rates using tracer gas decay, comfort parameters (percent relative humidity and temperature), culturable bacteria and culturable fungi. These variables were used to construct multiple linear regression models of indoor air quality outcomes. The regression equations for culturable bacteria and fungi will be presented in this paper.

The site of the study was in a coastal, temperate zone. Mean daily fall and spring temperatures are very similar (means 11.4ªC and 11.9ªC, respectively) with lower winter mean temperatures (daily mean 3.59ªC, $p<0.001$). The amount of rain is similar in fall and winter (seasonal daily means 8.4 mm and 9.5 mm respectively) with spring moderately but not significantly drier (daily mean 6.1 mm).

The schools were built between 1915 and 1995, with building construction styles including brick, wood frame, concrete block and pre-fabricated portables. Building maintenance was centralized in the school district and building custodians used centrally purchased cleaning materials. Ventilation systems were available in 78/116 (67%) of rooms studied and included central forced air, individual room ventilators, gas-fired heaters, or vane axial fans capable of either exhausting or supplying air. No rooms were equipped with air conditioning.

Bacterial bioaerosols:

The bacterial samples were collected using an Andersen N-6 sampling head with a flow rate of 28.3 liters per minute, using trypticase soy agar (BBL, Cockekysville, MD) culture medium (46 mL per 100x15 mm Petri plate). Samples were collected in duplicate as soon as the students left the room for the day. Outdoor samples were taken near the primary source of fresh air for the room. Plates were returned to the laboratory and incubated at 37ªC for 48 hours. Colonies were counted and identified to group by Gram stain. Micrococci and staphylococci were separated by glucose fermentation and staphylococci were tested for the presence of coagulase.

The overall geometric mean of the indoor bacterial samples was 226 CFU/m^3 with a geometric standard deviation of 2.75. The overall geometric mean of the outdoor bacterial samples was 26 CFU/m^3 with a geometric standard deviation of 2.56 (mean indoor counts greater than outdoor counts $p<0.005$). *Micrococcus* spp. were isolated from every indoor sample. In contract, *Bacillus* spp. were most commonly isolated from outdoor samples.

A multiple linear regression model was constructed as a predictive model of indoor bacterial concentration and is presented in Table I.

TABLE I: Multiple linear regression model of indoor bacterial concentration.

Variable	B	SE B	Beta	Significance	ΔR^2
Indoor CO2 (ppm)	0.001	0.592	0.423	<0.001	0.302
Indoor fungal concentration (ln CFU/m^3)	0.252	0.067	0.257	<0.001	0.096
Room volume (m^3)	0.005	0.001	0.253	<0.001	0.061
Exhaust flow (m^3/sec)	-0.882	0.342	-0.177	<0.05	0.026
Air exchange rate (ACH)	-0.204	0.071	-0.204	0.005	0.038
Signs of moisture (old stains)	0.349	0.140	0.172	<0.05	
Signs of moisture (current)	0.599	0.247	0.160	<0.05	0.030
Default = no signs of moisture					

Intercept (ln CFU/m3) = 1.44; Total R2 = 0.59

Also tried in the model but not retained were the variables describing: building age, building construction, number of occupants, amount of time windows/doors were open, indoor temperature and %RH, amount of carpeting, furnishings, barometric pressure, precipitation, wind speed and season.

Fungal bioaerosols:

Fungal samples were collected in a similar manner to the bacterial samples. Malt extract agar (BBL, Cockeysville, MD) was used as the culture medium. Plates were incubated for 7 days at room temperature for counting and identification of cultures by stereoscopic and phase contrast microscopy.

The overall geometric mean indoor fungal count was 323 CFU/m^3 with a geometric standard deviation of 2.81. The mean outdoor fungal count was 446 CFU/m^3 with a geometric standard deviation of 2.11. The top five indoor fungal cultures ranked by count were: sterile mycelia (isolated from 90% of samples) > *Penicillium* (96% of samples) > *Cladosporium* (98% of samples) > yeast (79% of samples) > *Aureobasidium* (67% of samples). Sterile mycelia are cultures that grow readily on malt extract agar, but do not sporulate. It was not possible to identify cultures without conidia or spore bearing structures. There were "blooms" of non-sporing fungi that contributed to counts placing this group first in the ranking. Although *Cladosporium* was present in slightly more samples, *Penicillium* was usually present in greater numbers. Outdoor counts were normally higher than indoor counts. The top five outdoor fungal cultures ranked by count were: sterile mycelia (isolated from 97% of samples) > *Cladosporium* (100% of samples) > *Penicillium* (88% of samples) > yeast (85% of samples) > *Aureobasidium* (85% of samples). *Botrytis*, *Aspergillus* and *Paecilomyces* were present in greater than 10% of samples both indoor and outdoor; thirteen additional fungal genera were identified, but were present in less than 10% of samples.

A multiple linear regression model was constructed. The strongest predictor of indoor fungi was outdoor temperature. Lower fungal counts were recovered in the winter. The variables of the model are presented in Table II.

TABLE II: Multiple linear regression model of indoor fungal concentration.

Variable	B	SE B	Beta	Significance	ΔR^2
Mean outdoor temperature (°C)	0.097	0.015	0.465	<0.001	0.203
Indoor bacterial concentration (ln CFU/m³)	0.300	0.071	0.294	<0.001	0.176
Building age < 1949 (non portable)	0.534	0.224	-0.229	<0.05	
Building age 1950 - 70	-0.467	0.196	-0.228	<0.05	
Building age > 1971	-1.646	0.290	-0.453	<0.001	0.096
Default = portable					
Exhaust flow (m³/sec)	1.073	0.341	0.211	<0.005	0.039
Windows open < ½ day	0.446	0.190	0.158	<0.05	
Windows open > ½ day	-0.096	0.146	-0.045	NS	0.026
Default = not open on sampling day					
Outdoor fungal concentration (ln CFU/m³)	0.257	0.098	0.184	<0.05	0.017
Mechanical ventilation (Y/N)	-0.364	0.150	-0.166	<0.05	0.020

Intercept (ln CFU/m³) = 2.32; Total R^2 = 0.61

Also tested in the model but not retained were variables describing: building construction, amount of time doors were open, season, indoor CO2 concentration, amount of carpeting, furnishings, indoor temperature, indoor %RH, precipitation, barometric pressure, wind speed, elevation above sea level, proximity to major arterial roads or treed parks.

CONCLUSIONS:

Indoor bacterial concentrations were influenced by presence and efficiency of room ventilation and were related to indoor fungal concentrations. Indoor fungal concentrations were influenced by outdoor temperature, age and type of building, presence of mechanical or use of natural ventilation and outdoor fungal concentration.

Variables commonly measured during an indoor air quality investigation can be used to understand bioaerosol concentrations. High commensal bacterial counts are clearly related to inadequate ventilation of the occupied space. In a similar manner, the use of mechanical or natural ventilation in buildings and the concentration of fungi in the outdoor environment is related to indoor fungal counts. Indoor fungal counts that substantively differ from the outdoor source flora require additional attention to building and environmental details. These data would suggest that indoor air quality investigations benefit from an integrated investigative approach.

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ANATOMY OF A FUNGAL PROBLEM

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ABSTRACT

Sampling was performed in a school with extensive visible fungal growth to evaluate exposure potential to building occupants. Musty odors and allergy-type symptoms were reported by building occupants. Initial testing using fungal sampling was not successful in identifying risks to building occupants. Additional sampling was conducted using total spore trap sampling, tape sampling and microbial volatile organic compound (MVOC) sampling. Analysis of the samples using total spore trap and tape sampling methods identified exposure potential to *Acremonium spp., Alternaria spp., Paecilomyces spp., Aspergillus niger* and others. Measurement of MVOCs showed that the indoor MVOCs were almost double the outside level. While sampling performed by traditional viable methods suggested no exposure potential, tape, spore trap and MVOC sampling data revealed greater exposure potential. Based on the results of this study, viable fungal sampling should not be the only method for conducting risk assessments relating to exposure to fungal spores and their bioeffluents.

KEY WORDS

Mold, Non-viable fungal growth, Microbial volatile organic compounds (MVOCs), Total spore trap sampling, Indoor air quality

INTRODUCTION

Significant fungal growth was identified on the underside of the gypsum roof deck in a Midwest Elementary School. Approximately 70% of the underside of the roof deck was visibly contaminated (Figure I). Traditional viable fungal sampling techniques including bioaerosol and Rodac contact samples on cultural media did not identify any significant growth. Due to this discrepancy between physical (visual) identification of fungal growth and analytical results, other methods had to be employed to further assess the potential risk to building occupants as spores that are not viable are still allergenic and possibly toxigenic even if they do not produce growth on culturable media (Carlson 1995)).

Core bulk samples of the roof structure (Figure II) were also collected to help determine if the fungal contamination had spread throughout the roof structure or was limited to the underside of the roof deck and insulation. This information was very critical, as it would help

FIGURE 1 Elementary School Mold Contamination.

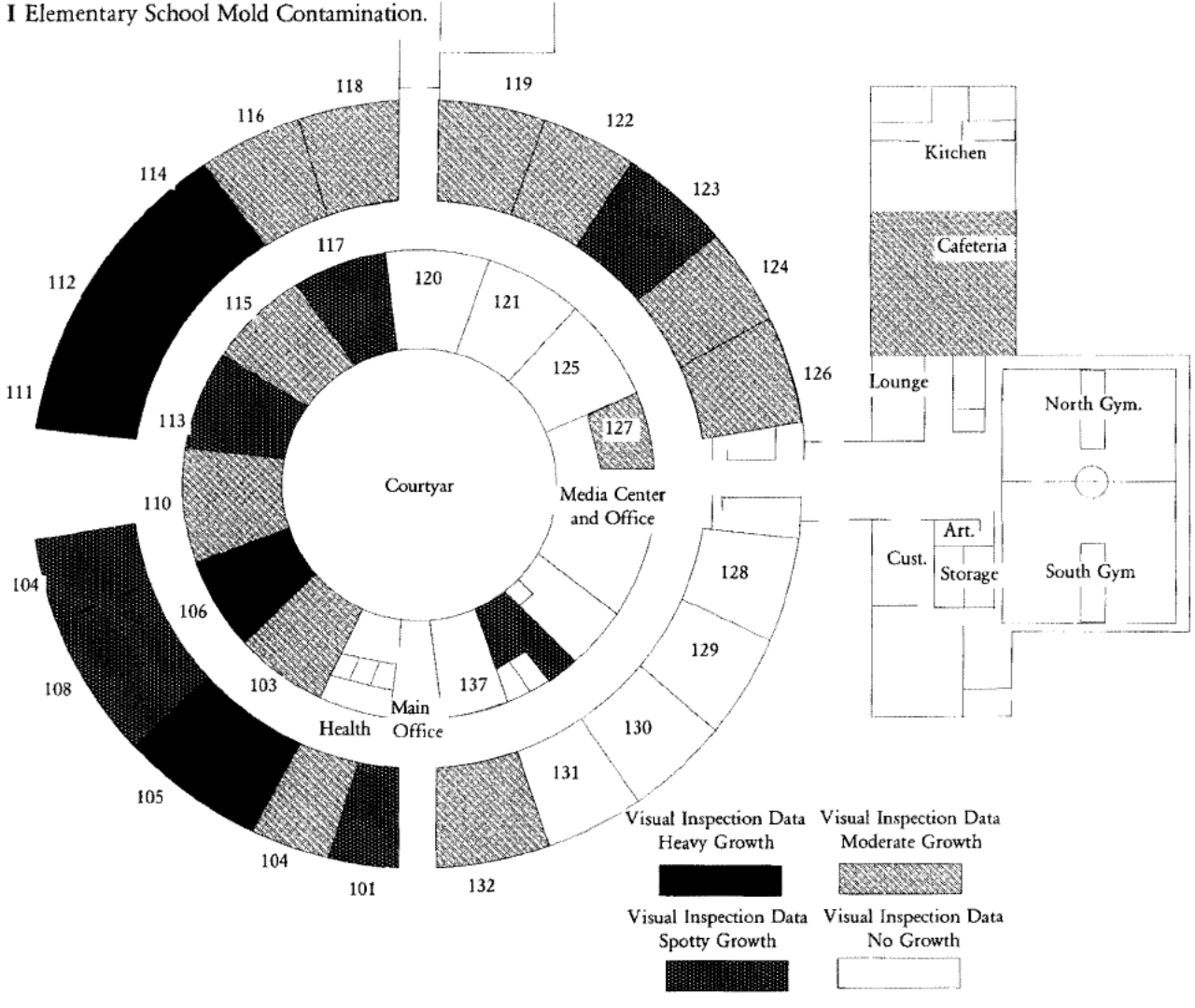
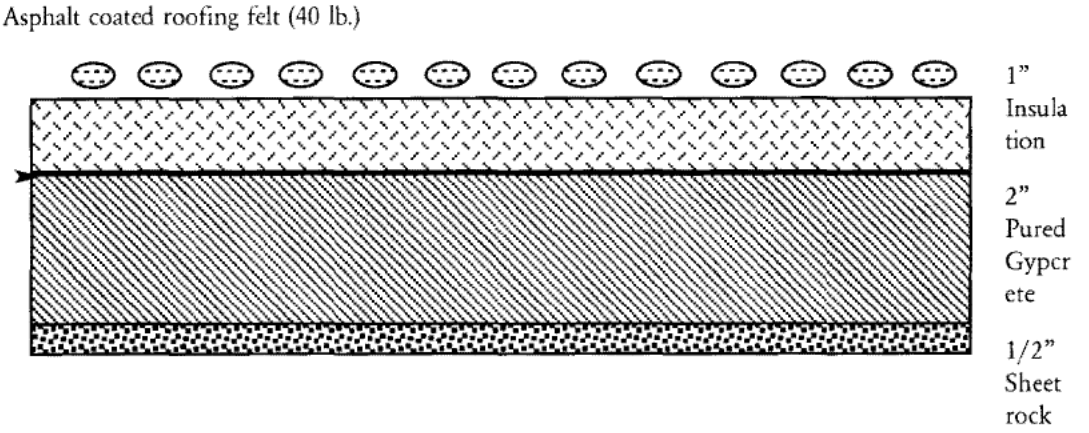


FIGURE II. Elementary School Roof Cross Section.



determine whether the entire roof structure would need to be replaced or if significant portions of the roof could be salvaged.

Direct tape samples of the visible growth were collected from the roof deck for microscopic identification. In addition, total spore trap sampling and MVOC testing was performed to provide a more realistic picture of the potential exposure to fungal spores (both viable and nonviable) and their metabolites.

MATERIALS AND METHODS

Bioaerosol culture sampling

Samples of airborne culturable fungi were collected using an Andersen impactor operating at a flow rate of 28.3 liters of air per minute for approximately 2-3 minutes. Culture media used during air sampling included DG-18, cornmeal (CMA), and mycological low pH (MLpH) agar (Dillon et al. 1996). Sampling was conducted during unoccupied conditions; however, normal activity was simulated and the HVAC system was operational (semi-aggressive sampling).

Contact Culturable Sampling

Rodac/contact plates and clear tape were placed on physical fungal growth. The plates were incubated at 25 degrees Celsius. Fungal organisms were identified by the adhesive tape technique. The adhesive tape is placed on the edge of a fungal colony and then placed on a slide with a small amount of 85% lactic acid mounting fluid. The gross morphology of the fruiting body is examined under a microscope.

Spore Trap Sampling

Samples of total airborne spores were collected with a personal Burkard sampler operating at 0.01 cubic meter of air per minute. Samples were pressed onto greased slides for times vary-

ing from 2 to 5 minutes. Slides were then examined at 400x and 1,000x and spore counts determined separately by morphological category.

MVOC Sampling

Air was sampled onto sorbent tubes containing Anasorb 747 (SKC Inc., Eighty Four, PA) at a total collection volume of 48 liters(L) (0.2L per minute for four hours). The sorbent was chemically desorbed with methylene chloride and analyzed by gas chromatography/mass spectroscopy (GC/MS). Total MVOCs is based on analysis of 12 specific volatile organic compounds (VOCs) known to be produced by microbiological growth (Air Quality Sciences, Inc., Atlanta, GA - Method AN055 for Analysis of VOCs, modified for MVOCs following AIHA field guide).

MVOC testing is relatively new in the United States; however, MVOC measures have been studied more extensively in Canada (Miller et al. 1988) and Scandinavia(Strom et al. 1994) for over 10 years.

RESULTS

Bioaerosol Culture Sampling

The outdoor control culture plate air samples were dominated by *Cladosporium* spp. (Table I) for both CMA and DG-18 agar plates. All the indoor concentrations were less than the outdoor samples with *Cladosporium* spp. generally the predominant species. This suggests that overall exposure to viable fungi indoors was less than outdoors. The low overall numbers in the samples is generally typical of non-problem buildings and a definitive interpretation from these samples was not possible.

TABLE I: Concentrations and dominant organisms of culturable fungi in air samples.

Location	Agar Type	Total Concentration CFU/m³	Dominant Organism
Media Center	DG-18	424	*Cladosporium* spp.
	CMA	448	*Cladosporium* spp.
Room 118	DG-18	225	*Cladosporium* spp.
	CMA	283	*Cladosporium* spp.
Room 116	DG-18	366	*Cladosporium* spp.
	CMA	260	*Cladosporium* spp.
Room 114	DG-18	202	*Cladosporium* spp. *Aspergillus versicolor*
	CMA	72	*Penicillium* spp.
Room 114 in ceiling plenum	DG-18	165	*Cladosporium* spp.
	CMA	214	*Penicillium* spp.
Outdoors	DG-18	1,367	*Cladosporium* spp.
	CMA	1,113	*Cladosporium* spp.

Contact Cultural Sampling

Rodac contact samples collected from visible sites of fungal growth on the underside of the roof deck identified significant viable growth on only one sample. At this point, however, the analyst noticed heavy non-viable deposition of desiccated *Alternaria* spp., *Conidia*, and *Mycelia* on the Rodac contact plates (TABLE II).

TABLE II: Concentrations and organisms of culturable fungi on Rodac samples.

Location	Agar Type	Total Concentration CFU/plate	Primary Organisms
Room 105 - ceiling (purple growth)	DG-18	ND	
Room 114 (black growth)	DG-18	Overgrowth	*Cladosporium* spp. *Paecilomyces* spp.
Room 114 (no visible growth)	DG-18	ND	--
Room 116 (orange growth)	DG-18	2	*Aspergillus niger* *Aspergillus fumigatus*
Room 118 (no visible growth)	DG-18	2	Yeast
Room 114 - floor tile	DG-18	11	*Cladosporium* spp.
	MLpH	14	*Cladosporium* spp. Non-sporulating Mycelia

TAPE SAMPLING

Tape slide samples of various areas of physical fungal contamination were collected. The analysis of these tape slides confirmed the presence of significant fungal contamination of the underside of the roof deck. The dominant species identified included *Acremonium* spp., *Alternaria spp.* and *Cladosporium spp.* (Table III).

TABLE III: Qualitative analysis of tape slide samples from underside of roof deck.

Location	Amount of fungal Growth	Primary Organisms
Room 105 - dark growth	Heavy	*Acremonium*
Room 114 - dark growth	Heavy	*Cladosporium* spp. *Alternaria* spp *Penicillium* spp.
Room 114 - orange growth	Heavy	*Cladosporium* spp. *Alternaria* spp. *Penicillium* spp.
Room 132 - dark growth	Heavy	*Alternaria* spp.
Room 103 - dark growth	Heavy	*Alternaria* spp. *Acremonium* spp.
Room 107 - dark growth	Heavy	*Alternaria* spp. *Acremonium* spp.
Room 107 - brown growth	Heavy	*Alternaria* spp. *Cladosporium* spp.
Room 115 - dark growth	Heavy	*Alternaria* spp. *Cladosporium* spp.
Room 118 - dark growth	Heavy	*Alternaria* spp.
Room 122 - dark growth	Heavy	*Alternaria* spp.
Room 126 - dark growth	Heavy	*Alternaria* spp. Acremonium spp.
Room 127 - dark growth	Heavy	*Alternaria* spp. *Acremonium* spp. *Cladosporium* spp.
Cafeteria	Heavy	*Alternaria* spp.
Room 114 - control sample no growth	No spore colonization	--

Spore Trap Sampling

The spore trap results suggests that exposure to total fungal spores was different indoors compared to the outdoors (Table IV). The dominant spores identified outdoors included *Cladosporium spp.* and *Ascospores*. However, the dominant species identified indoors in all the samples were *Penicillium spp.* and *Aspergillus spp.* The exception was the indoor control sample in the media room which identified *Cladosporium spp.* (similar to the outside) as the dominant species. Microscopic examination of the spore trap samples suggested that exposure to fungal spores of *Penicillium spp.* and *Aspergillus spp.* was occurring within the building. These results were also significantly different from the bioaerosol culturable sampling that identified *Cladosporium spp.* as the dominant indoor fungi.

TABLE IV: Concentrations and dominant organisms of total spores.

Location	Concentration Spores/m³	Dominant Organisms
Room 111	60	*Penicillium* spp. *Aspergillus* spp.
Room 114	360	*Penicillium* spp. *Aspergillus* spp.
Room 107	560	*Penicillium* spp. *Aspergillus* spp.
Room 116	1,860	*Penicillium* spp. *Aspergillus* spp.
Media Room - indoor control	320	*Cladosporium* spp.
Outdoors	6,360	*Ascospore*
Outdoors	2,340	*Cladosporium* spp.

Bulk Sampling

Bulk core samples were collected of the roof to determine the extent of the contamination and to determine if the major portions of the roof structure could be salvaged. The samples were analyzed through the dilution method. The results of the samples found that the 1" roof insulation was heavily contaminated with *Acremonium spp.*, as was the asphalt coated roofing felt. The 2" poured gypcrete was found to contain no viable fungal growth (TABLE V). Therefore, salvaging of the gypcrete roof deck was possible.

TABLE V: Concentration and dominant organisms in roof core samples - analyzed by dilution method.

Location	Agar Type	CFU/Gram	Primary Organisms
Dry roof insulation	CMA	<870	No growth
	MEA	<870	No growth
Wet roof insulation	CMA	4,555,556	Acremonium spp.
	MEA	4,415,385	*Acremonium* spp.
Wet roof felt	CMA	1,052,212	*Acremonium* spp.
	MEA	1,342,478	*Acremonium* spp.
Gypcrete core	CMA	<855	No growth
	MEA	<855	No growth

MVOC Sampling

MVOC samples were taken from three indoor and one outdoor locations. The outdoor sample acted as a control. MVOCs were detected in all indoor samples at a concentration almost double of the outdoors (Table VI). The outdoor sample isolated only one compound - 2-Octen-1-ol; however, the indoor samples identified other compounds not found in the outdoor sample - 3-Octanol and 1-Octen-3-ol. The fungal contamination of the roof deck was potentially compromising the indoor air quality of the school.

TABLE VI: Individual and total MVOC levels.

Location	Total MVOC ng/m³	Compounds Identified	Concentration ng/m³
Room 112	12,393	2-Octen-l-ol	8,597
		1-Octen-3-ol	2,248
		3-Octanol	1,549
Room 114	12,129	2-Octen-l-ol	8,475
		1-Octen-3-ol	2,202
		3-Octanol	1,452
Media Center	10,198	2-Octen-l-ol	7,877
		1-Octen-3-ol	2,321
Outdoors	6,817	2-Octen-l-ol	6,817

DISCUSSION

Based on the findings of the investigation, the following changes were implemented:

- The underside of the roof deck was decontaminated under controlled conditions using a 5-10% bleach solution. After the decontamination was completed and the roof deck was dry, The surface was encapsulated with a mold inhibitor (Fosters 40-20).
- The roof was removed down to and including the existing insulation material and the gypcrete was visibly inspected. Any water damaged roof material was removed at this time and any remaining tar surface was decontaminated. The indoor enclosure was intact during the roof removal and re-roofing phase.
- After all the remediation was completed and the new roof installed, the indoor enclosure was removed and the interior of building was disinfected using appropriate cleaning and industry standard disinfectant procedures.
- The ceiling plenum was ventilated to ensure that stagnant moist air does not lead to microbial regrowth.

CONCLUSIONS

The following conclusions are based on the visual inspection and the various testing conducted:

- Fungal contamination was present in almost 70% of the classrooms along with the hallway, gymnasium and cafeteria. Species isolated by tease tape samples confirmed the presence of both allergenic (able to cause allergy symptoms such as mucous membrane irritation, stuffy/runny nose, cough, etc.) and potentially toxigenic (able to cause injury to biological tissue usually with high dose exposures) species. These species can be a health concern (irrespective of whether the spores are viable or non-viable) to sensitized individuals especially in the quantities isolated within the building structure.
- Microbial VOCs indoors were significantly greater than the outdoors pointing to the "off-gassing" of irritants from the existing fungal contamination.
- The viable fungal growth in the roof was limited to the roof insulation, the felt immediately underneath the insulation and the underside of the roof deck. No significant contamination was isolated in the gypcrete.
- The source of the moisture leading to the fungal contamination of the roof deck was a combination of roof leaks and condensation.

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CHARACTERIZATION OF BIOAEROSOL EMISSIONS FROM A SUBURBAN YARD WASTE COMPOSTING FACILITY.

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ABSTRACT:

During the months of September through November, 1995, extensive bioaerosol measurements were made at and around at composting facility over a 10 day sampling period. Airborne bacterial concentrations were much higher on-site than off site and off site bacterial concentrations were significantly higher downwind of the composting facility. Airborne fungal concentrations were not significantly higher closer to the compost facility than further off-site. Particulate, endotoxin and beta 1,3 glucan concentrations were significantly higher on-site than off-site.

KEY WORDS:

Composting, yard waste, fungi, bacteria, endotoxins, glucans, agricultural dust.

INTRODUCTION:

Large scale yard waste composting is becoming more common due to laws banning the burning or landfilling of yard waste. Only a few studies have investigated the possible health effects of yard waste composting. Following complaints and concerns of residents, extensive bioaerosol measurements were made in a yard waste composting facility and adjacent areas in Lake Forest, Illinois in order to characterize bioaerosol emissions from the composting site.

METHODS AND MATERIALS:

Samples were collected on 10 days from September to November, 1995. Bioaerosol measurements were made both at various points at the composting site (upwind, downwind, at com-

posting house, and workers personal sampler) and at 4 community sites located about 300 meters from the composting facility.

Andersen impactor sampling was used to measure viable fungi and bacteria. Standard malt extract media was used for fungi collection and soybean casein media was used for total bacteria sampling. These Andersen samplers collected 10 to 50 liters of air over short sampling time (less than 2 minutes). Andersen samples were collected over a several hour period in the morning at 8 sites. If time permitted, the Andersen samplers were then autoclaved and additional Andersen sampling was conducted at several sites in the afternoon. The Andersen plates were then cultured at 4 to 6 days at 25°C, and the fungal and bacterial colonies counted and divided by volume of air sampled to calculate cfu/m^3 Fungal colonies were then identified by standard microscopic methods (Samson et al. 1996) and bacterial colonies underwent Gram staining.

Fungal spores were collected by a Kramer-Collins sampler over a period of 6 to 8 hours. Airborne dust samples were collected on quartz filters over a 6 to 8 hour sampling period to measure total particulates, beta-D-glucans, and endotoxin concentrations. In addition, weather conditions and activities at the composting site (pile turning, etc.) were recorded.

RESULTS:

Concentrations of viable fungi and bacteria are listed below in Table I.

Note that bacterial concentrations were highest just downwind of the composting piles.

TABLE I: Summary of Andersen Sampling for Viable Bacteria and Fungi.

Location	Number	Fungi Samples cfu/m^3			Bacteria Samples cfu/m^3		
	of samples	Mean	Median	Range	Mean	Median	Range
4 Sites about 300 m from compost facility	55	12,355	5,840	80- 94,230	3,109	2,072	160- 5,600
1 m downwind piles	18	3,657	2,400	480- 17,680	19,044	6,840	640- 78,880
10 m downwind	18	3,098	2,360	480- 13,680	9,783	4,640	480- 41,600
Fenceline downwind	17	17,871	5,120	960- 97,560	5,915	4,440	640- 19,120
10 m upwind	18	2,448	2,230	480- 5,280	6,800	4,880	560- 26,240
Fenceline upwind	18	8,440	4,800	80- 57,680	4,044	3,880	320- 8,400

The most common fungal genera cultured on the Andersen plates from the composting site were *Aspergillus* (45% of total fungi), *Penicillium* (21%), *Cladosporium* (8%) and *Alternaria* (5%). Off-site the most common fungi cultured on Andersen plates were *Aspergillus* (64%), *Penicillium* (16%), *Cladosporium* (5%) and *Alternaria* (4%). In general, no large differences in composition of fungal genera were seen between upwind and downwind samples.

Concentrations of particulates, endotoxins and beta-glucans are reported below in Table II. Note that levels of particulates, endotoxins and beta 1,3 glucans were considerably higher on-site than off-site.

TABLE II: Summary of Off site and On site values for total particulates, endotoxins and glucans.

Exposure	28 off-site samples			18 on-site samples		
	Mean ng/m³	Median ng/m³	Range ng/m³	Mean ng/m³	Median ng/m³	Range ng/m³
Total particulates	98,892	84,000	41,000- 278,000	631,222	384,000	47,000- 1,805,000
Endotoxins	0.14	0.14	0.01-0.41	1.94	1.99	0.12- 6.06
Beta 1-3 glucans	0.24	0.13	0.01-0.78	2.17	0.79	0.12- 14.45

Mean Kramer Collins total spore concentrations were 13,451 spores/m³ on-site (range 5,223-26,067 spores/m³) and 8,772 spores/m³ off-site (range 243- 18,276 spores/m³).

Table III below compares concentrations of bioaerosols and particulates on days with no site activity and days with site activity.

TABLE III: Comparison of bioaerosol and particulate concentrations during periods of activity and periods of no activity. Includes both on and off site samples.

Exposure	Mean- Periods of no activity	Mean- periods of compost activity (turning etc)	P Value- t-test with unequal variances.
Total particulates ug/m³	413	282	0.51
Total fungi cfu/m³	2,793	8,926	0.74
Total bacteria cfu/m³	2,293	8,816	0.002
Beta 1-3 glucans ng/m³	0.62	1.08	0.42
Endotoxins ng/m³	0.18	1.01	0.003

Spearman coefficients were run to analyze if the wind direction from compost site had any correlation with off-site bioaerosol concentrations. Downwind off-site locations were found to have significantly higher bacterial concentrations than upwind sites.

DISCUSSION AND CONCLUSIONS:

The compost facility was a major source of airborne bacteria, with bacteria concentrations averaging 19,044 cfu/m³ 1 meter downwind from piles, 9,703 cfu/m³ 10 meters downwind of piles, and 5,915 cfu/m³ downwind at compost facility fenceline (Table I). Compost facility activity significantly increased downwind concentrations of bacteria both on-site and off-site. In future studies, speciation of bacteria emitted from composting operations might yield interesting data.

On-site concentrations of endotoxin on-site sometimes exceeded 4 ng/m³ (Table II), a threshold in which Milton et al. (1996) reported significantly decreased lung function in fiberglass

workers. Compost operators should wear respirators when engaging in dust generating activities in order to reduce exposure to endotoxins and total particulates.

The compost facility did not appear to be a major source of airborne fungi. The relatively high concentrations of total viable fungi and total viable *Aspergillus* found on and off the composting site may be partially due to the warm and wet summer and fall season of 1995 in the Lake Forest region. This compost facility was located a suburban area which contained forests and wetlands which might be major sources of bioaerosols. A study of a yard waste facility in Islip, New York (Boehler et al. 1994) reported only a modest association between wind direction and bioaerosol concentrations. Marchard et al. (1995) measured bioaerosol concentrations at a municipal solid waste recycling plant and found very high concentrations of viable airborne bacteria (mean of 141,000 cfu/m^3 and range 8,700 to 528,600 cfu/m^3) but modest concentrations of airborne viable fungi (mean of 4,700 cfu/m^3 and range of 700 to 7,200 cfu/m^3). On the other hand, Ekrich et al. (1995), reported much higher concentrations of both fungi and bacteria at a compost facility as compared to background. Total median fungal concentrations (in cfu/m^3) were 24,000 at the composting facility, 1,200 at 40 meters downwind and 700 at a background site. Total median bacterial concentrations (in cfu/m^3) were 56,000 at the composting facility, 2,400 at 40 meters downwind and 230 at a background site.

Much more research is needed to characterize bioaerosol emissions from composting facilities and their potential health effects. The type of yard waste composted, weather, topography, and the specific construction and operating practices of composting facilities all may play a major role in determining bioaerosol emission rates.

ACKNOWLEDGEMENTS:

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AIRBORNE CONTAMINATION OF EASTERN CANADA SAWMILLS.

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ABSTRACT:

The purpose of this study was to quantify and identify the airborne contamination in sawmills in order to monitor the exposure levels for workers. Seventeen sawmills were chosen to cover a wide range of size, geographic distribution, wood species and, within each sawmill, different work areas (debarking, sawing, sorting, or planing) were studied separately. Microbial contaminants were assessed using all glass-impingers-30 and six-stages Andersen samplers. Tryptic soy agar was used for bacteria, Rose Bengal Agar (RB) was used for culturable molds (30°C) determination. PVC filters were used at a flow rate of 2l/min for at least 6 hours for dust level measurements. Endotoxins were measured in AGI-30 liquid. Temperature and humidity were also measured. *Penicillium* species were the most predominant molds. We identified up to 40 different *Penicillium* species in these environments. Debarking was the working site most highly contaminated by molds, bacteria and endotoxins (p=0,0001). At this working site mold levels reached up to $1.5x10^6$ CFU/m^3, the culturable bacteria levels were in average 3.3 X105 ± 3.1 $X10^5$ CFU/m^3 and the median value for endotoxin level was 1081 endotoxin units/m^3. Planing site was the most highly contaminated with dust (median: 3.0 mg/m^3) ($p<0.05$). Eastern North America sawmills contain airborne biological contaminants which vary between working sites and their microflora is different from those previously described in European sawmills.

KEYWORDS

Aerobiology, Sawmills, Environmental Biological Exposure, Molds, Endotoxins, Wood Dust.

INTRODUCTION

Sawmills are an important industry in Eastern North America. In the Province of Quebec, up to 12 000 workers are directly involved in this industry. Health risks associated with working in sawmills include lung cancer (Flechsig et al. 1990) and respiratory diseases related to inhalation of organic dusts (Belin, 1987; Wimander et al. 1980) have been reported.

To our knowledge, no published study was performed in Eastern North America to analyze the airborne contamination in sawmills. Since the tree species as well as the climate are very dif-

ferent from European countries, we cannot use these data to evaluate the potential respiratory risk to eastern north american workers. Spruce and fir account for the majority of timber processed in eastern north american sawmills. Some other tree species, such as pine, cedar, birch or oak are also used but these species are only rarely processed.

Different geographical areas divide Eastern North America. The weather conditions including seasonal variations may influence the exposure related to wood spoilage, dust production, and tree microflora. The size of the sawmill may also influence the environmental exposure among workers: the bigger sawmills may be better ventilated but the production rate may be higher, which could influence worker's exposure.

The major aims of this study were: 1) to evaluate environmental biological content in sawmills, 2) to verify the influence of working sites and 3) to give detailed description of the sawmill fungal microflora and other major antigens.

MATERIAL AND METHODS

SAWMILLS SELECTION

We evaluated the air contamination of 17 sawmills in 4 different geographical regions ot the province of Quebec (named A, B, C and D): Region A) South-East, B) South-West, C) the North-West, and D) the North-East.

We analyzed air quality in 12 Spruce/Fir sawmills and with 5 other tree species including: 2 birch,1 oak, 1 cedar, and 1 pine sawmills.

Each of work sites, debarking, sawing, planing and sorting, were evaluated separately.

ENVIRONMENTAL SAMPLING: INHALABLE DUST (DRY WEIGHT)

Pre-weighed 25 mm PVC filter (0,8 µm) housed in IOM open-faced cassettes (SKC, Eighty Four, Pennsylvania) were used with SKC 224-44XR personal sample pumps (Dur-Pro, Brossard, Canada) calibrated at 2.0 l/min (Kurz flow-meter). For technical reasons, the dust values are not available for the first seven sawmills.

Environmental analyses: culturable microorganisms and endotoxins

Culture media

Tryptic-soy agar (TSA) (Difco, Detroit, USA) supplemented with cycloheximide (500 mg/l) was used for the isolation of total mesophilic bacteria. Rose Bengal Agar (RB) (Difco), Czapek Solution Agar (CZA) (Difco) and Sabouraud Dextrose Agar (SDA) (Difco), all of these supplemented with chloramphenicol (50 mg/l), were used for molds culture.

Air sampling

Air samples were taken on a table, 1m above the floor, as near the working area as possible. When possible, all doors were kept closed and the sampling apparatus were kept far from ventilation sources. Two types of air samplers were used at each sampling site: 3 six-stage Andersen impactor (Grasby Andersen, Atlanta, GA, USA) and 3 all glass liquid impingers-30 (AGI-30) (Ace Glass Inc., Vineland, NJ, USA). The Andersen impactors were used at a sampling flow of 28.3 l/min. They were loaded with TSA for 1 minute sampling (bacteria) and with

RB, or SDA, or CZA for a 30 seconds sampling (molds). The 3 AGIs contained 20 ml of sterile saline solution (0,85% NaCl) and used at 12.5 l/min for 16 minutes. Control samplings were performed outside 1 to 5 km upwind from the sawmill.

Samples analysis

The TSA from Andersen samplers were incubated 30°C for culturable bacteria.

The samples from AGIs were measured (to evaluate evaporation) and recovered. The impingers were then washed with a volume of sterile saline solution containing 0.1% Tween 80 to complete the sample volume to 30 ml. The final sample contained about 0.03% Tween 80. One milliliter of this 30 ml volume was diluted in 9 ml of sterile saline solution (10^{-1}) and this was repeated to a 10^{-5} dilution. Each sampling suspension (3/site) was diluted in triplicates. One hundred microliters of the dilutions (10^{0} to 10^{-3}) were plated on different media appropriate for the microorganism to be studied (see culture media section) as well as 0.5 and 1 ml of the non diluted AGI liquids. The culture conditions were the same as the Andersen samples conditions.

For Andersen and AGI samples, bacteria were counted after 48 hours, thermophilic actinomycetes after 5 and 7 days and molds and yeasts counted after 7 and 10 days. All macroscopically different mold colonies were isolated for later identification. For *Penicillium* species, John I. Pitt's guide for *Penicillium, Talaromyces* and *Eupenicillium* genera identification was used (Pitt, 1979).

Endotoxins from AGIs samples were measured using the Limulus Amoebocyte Assay (LAL) endpoint chromogenic test (Associates of Cape Cod, Woods Hole, Ma). Samples were diluted and a inhibition/enhancement test was performed prior to measurement. Controls were obtained with sterile saline water with which sterile AGI-30 sampler was washed for a few minutes

STATISTICAL ANALYSIS

Statistics were performed to compare the 12 Spruce-Fir sawmills. Since the other 5 sawmills used different wood species no statistics were possible for these data. When normality distribution was not encountered, rank tests were performed to compare the different values and median values as well as range were given. When normality or log normality was encountered, arithmetic mean and standard deviation was given. A p value <0.05 was considered significant.

RESULTS

Figure I and II show the culturable bacteria (recovered on TSA) and molds (on Rose Bengal agar) levels (CFU/m^3). Figure III shows airborne inhalable dust (mg/m^3) (dry weight) and figure VI shows endotoxin levels (EU/m^3) measured from AGI-30 liquid. Expressed values are mean ± SD. Each working site is presented separately. Missing data mean that this site was nor present or sampled in the sawmill.

In the 12 Spruce/Fir sawmills, debarking sites were the most highly contaminated with molds ($p=0.0001$), bacteria and endotoxins ($p<0.05$).

FIGURE 1: Mesophillic bacteria (CFU/m³) (mean±SD) for debarking, sawing, sorting and planing sites. SF=Spruce/Fir; BI=Birch; PI=Pine; CE=Cedar; OA=Oak.

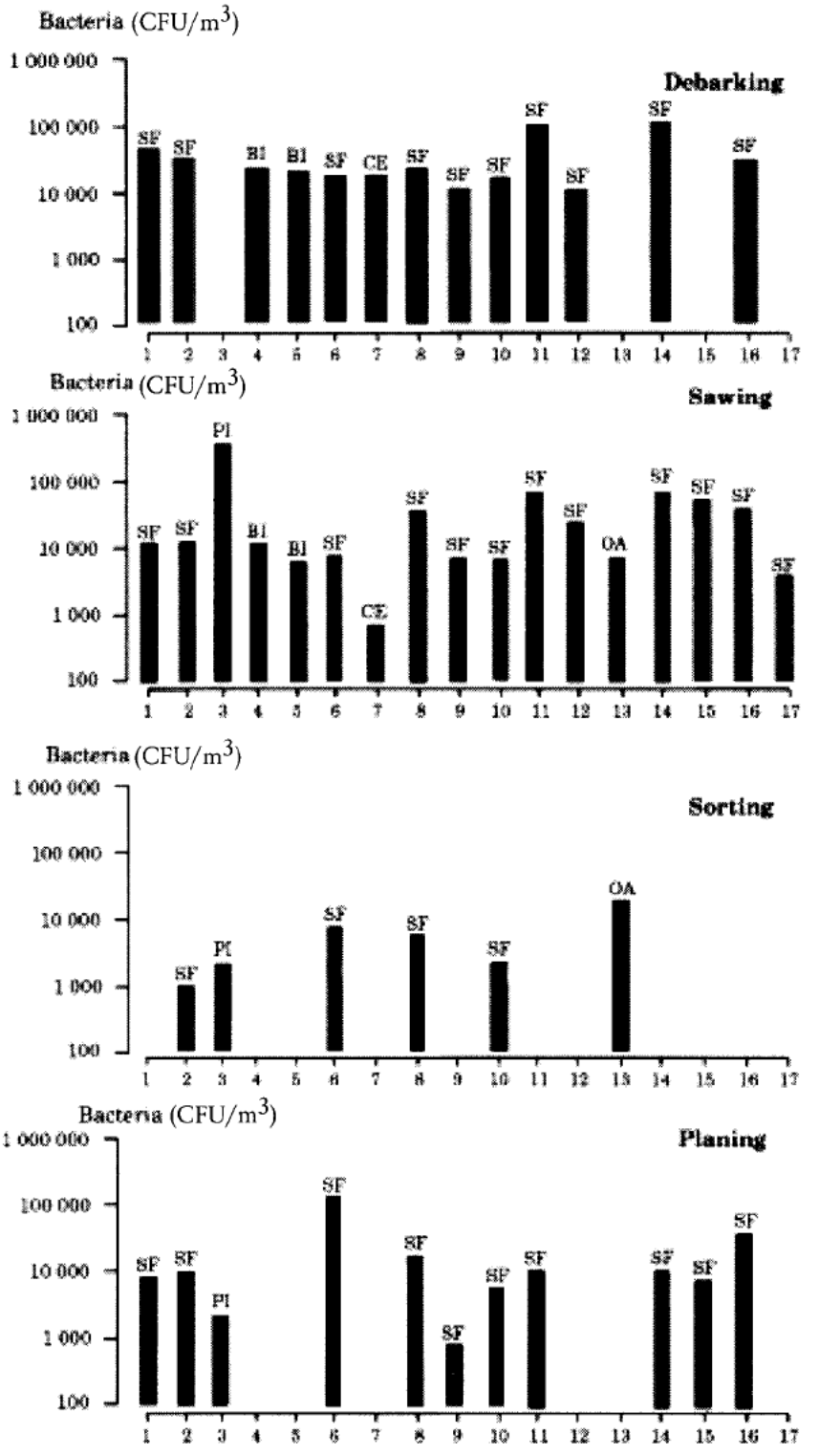

FIGURE II: Molds recovered on Rose bengal agar (CFU/m³) (mean±SD) for debarking, sawing, sorting and planing sites. SF=Spruce/Fir; BI=Birch; PI=Pine; CE=Cedar; OA=Oak.

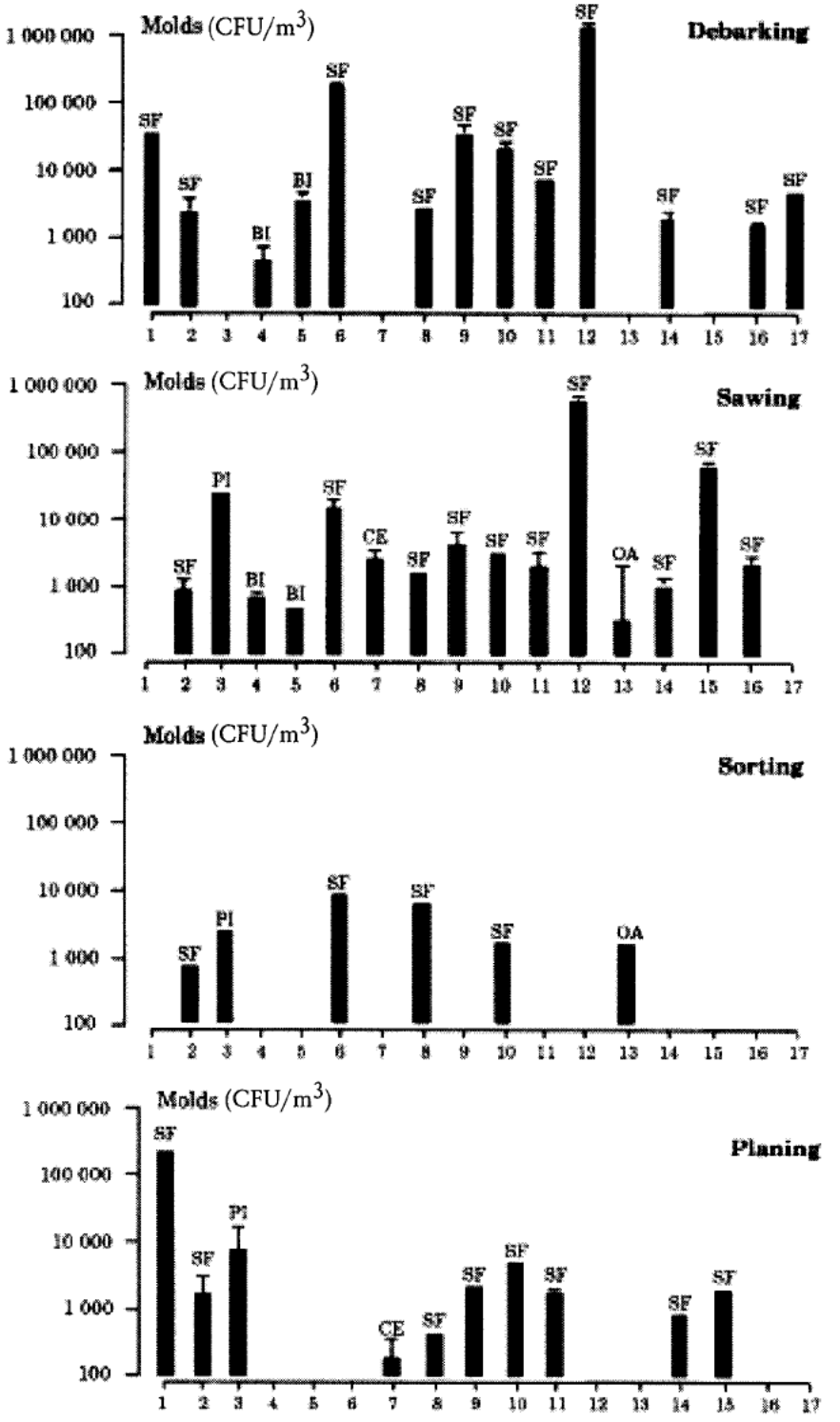

FIGURE III: Inhalable dust (mg/m³) (mean±SD) for debarking, sawing, sorting and planing sites. SF=Spruce/Fir; BI=Birch; PI=Pine; CE=Cedar; OA=Oak.

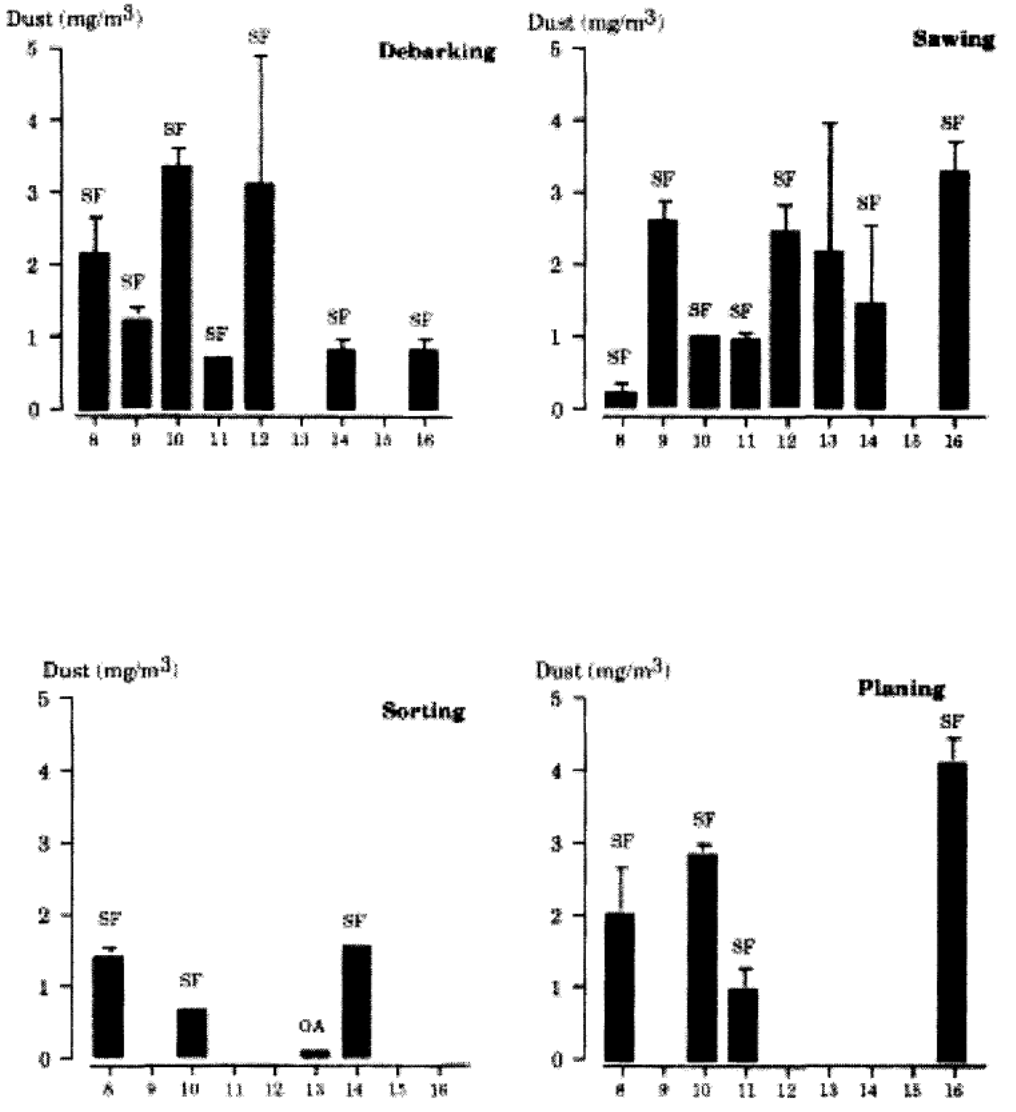

FIGURE IV: Endotoxins (Endotoxin Units/m^3) (mean±SD) for debarking, sawing, sorting and planing sites. SF=Spruce/Fir; BI=Birch; PI=Pine; CE=Cedar; OA=Oak.

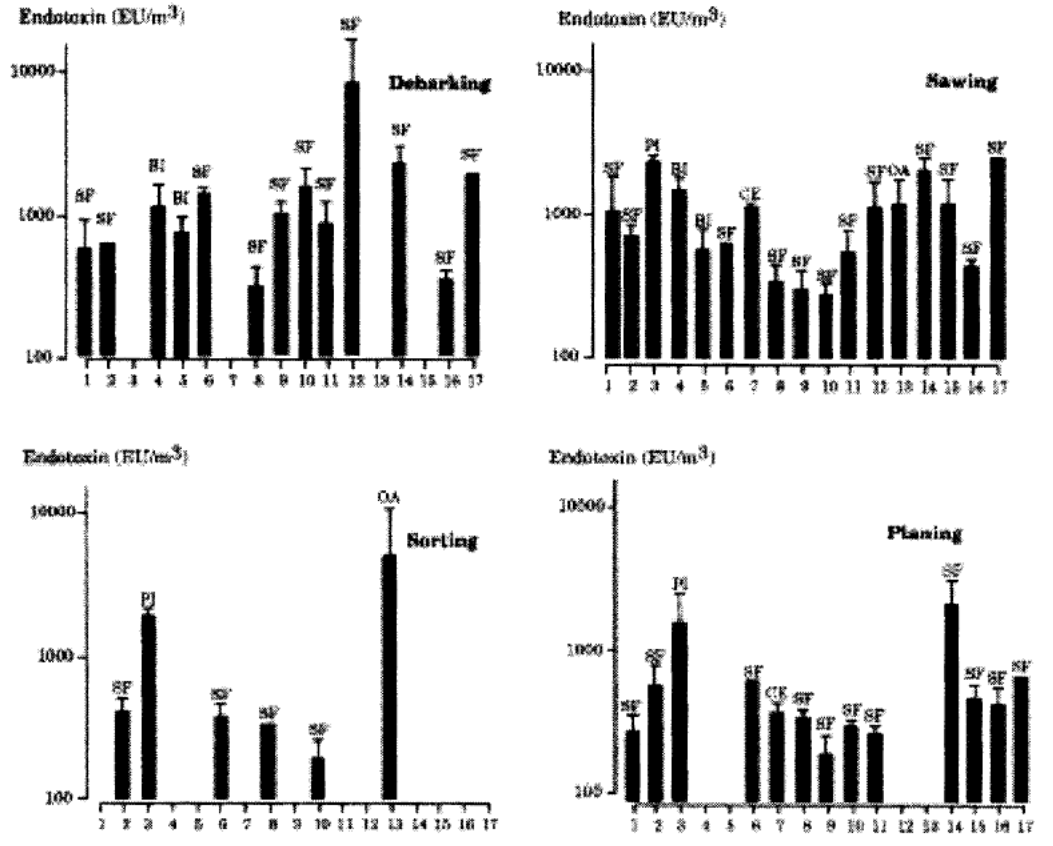

Table I shows the mold species and genera identified in the different sawmills from the 3 culture media.

TABLE I: *Penicillium* species and other taxa recovered from sawmill air samples.

Penicillium **species**	**Other taxa**
P. aculeatum	*Aspergillus sp.*
P. aurantiogriseum	*Asp. fumigatus*
P. canescens	*Asp. flavus*
P. chrysogenum	*Asp. niger*
P. citreonigrum	*Asp. terreus*
P. crustosum	*A. versicolor*
P. echinulatum	*Acremonium butyri*
P. fellutanum	*Acremonium sp.*
P. glabrum	*Alternaria sp.*
P. islandicum	*Arthrinium sp.*
P. janczewskii	*Baueveria sp.*
P. janthinellum	*Chrysonilia sp.*
P. jensenii	*Cladosporium sp.*
P. lignorum	*C. carionii*
P. lividum	*Eurotium sp.*
P. melinii	*Exophiala sp.*
P. mineoluteum	*Fusarium sp.*
P. montanense	*Geotrichum sp.*
P. myczinski	*Lecythophora sp.*
P. novae-zeelandiae	*Paecilomyces sp.*
P. oxalicum	*Phialophora sp.*
P. piceum	*Phoma sp.*
P. purpurescens	*Rhizopus sp.*
P. purpurogenum	*Sporothrix sp.*
P. raistrickii	*Trichoderma sp.*
P. roquefortii	*Verticillium sp.*
P. roseopurpureum	
P. rugulosum	
P. simplicissimum	
P. spinulosum	
P. thomii	
P. variabile	
P. velutinum	
P. verrucosum	
P. viridicatum	
P. waksmanii	
Penicillium sp.	
Eupenicillium sp.	

DISCUSSION

In Eastern Canada sawmills, molds belonging to the genera *Penicillium* were by far the most predominant. *P. myczinskii, P. fellutanum, P. spinulosum* and species showing teleomorph state belonging to *Eupenicillium* sp. were very important contaminants.

The nature of the contaminants found in the Eastern Canadian sawmills seems very different to those reported in Europe where *Rhizopus* sp., *Paecilomyces* sp were frequent while in our study very few *Rhizopus* sp. and *Paecilomyces* sp. were found. In the sawmill #4 (Birch sawmill), *Rhizopus* sp. was the most important mold but the levels found were very low (data not shown). Interestingly, *Paecilomyces* sp. was especially important in non Spruce/Fir sawmills. Since only few studies have been performed in Spruce-Fir sawmills, the difference in the nature of airborne microflora may be explained by the difference in tree species.

The most highly mold contaminated sawmill was a Spruce-Fir (#12). In this sawmill, *P. myczinskii* was the most important and represented up to 46% of the fungal contaminants (data not shown). The sorting sites were the less contaminated with inhalable dust while planing sites showed the highest dust levels. Debarking and sawing sites showed dust levels slightly lower than planing sites. The high endotoxin levels found were surprising since no such levels have been described in the previous studies. Exposure to high endotoxin levels may be harmful to the workers.

CONCLUSION

North America sawmills contain airborne biological contaminants which vary between working sites and their microflora is different from those previously described in European sawmills. The workers are exposed to high levels of bacteria, molds and endotoxins. A health study among sawmills workers is now in process to verify any effects of the exposure to this environment.

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PREVALENCE OF FUNGI IN CARPET DUST SAMPLES

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ABSTRACT

Samples of dust collected from carpeting can provide an indication of the microbial condition of a building with the carpet material having potential to be both an amplifier (growth site) and reservoir for fungi. Interpretation of the data obtained from analysis of the dust is helpful when making a determination as to the overall condition of both the building and the carpet material. The authors have collated data from 243 samples collected in both problem and control buildings across the USA. A comparison is made between the results obtained from both problem and control buildings, with particular emphasis on the biodiversity found and the regional differences observed. The study concludes that despite the ubiquitous nature of fungi there are a number of key indicators which can be used to determine if the dust from a carpet suggests a contaminated building.

KEY WORDS:

Fungi, Carpet Dust, *Aspergillus*, Fungal Contaminated Buildings

INTRODUCTION

Between June 1995 and May 1998, 243 dust samples were collected from carpets in 35 buildings for fungal analysis. The 35 buildings included in this review covered a range of commercial, institutional, and governmental establishments including offices, schools, hospitals, and hotels. Geographically the buildings are in three major regions: Northeastern United States (16 buildings), Southeastern United States (14 buildings), and the Pacific Northwestern United States (five buildings). The number of samples collected from each building varied depending on the requirements of the evaluation.

Samples were categorized as coming from either problem buildings or control buildings. Problem buildings were defined by one or more of the following criteria: occupants have medically diagnosed health problems related to fungi contamination, water damage to the buildings, high humidity in the occupied spaces, or moldy odor problems in the building. Control buildings were defined as either the control buildings used for comparative purposes during an evaluation of a problem building, or problem buildings that had been successfully remediated of fungal contamination (post-remediation samples).

Of the thirty five buildings sampled, thirty were classified as problem buildings, and five were classified as control buildings, of which three had previously been problem buildings and had been remediated.

Results of carpet dust samples were selected for this study because the carpet in problem buildings can be a reservoir of fungal spores that have aerosolized from other contaminated materials and settled out with dust into the fleecy surface of the carpet fibers. Carpet in problem buildings can also be an amplification (growth) site if the carpet has been flooded or subject to enough moisture to increase water activity to the point where fungal growth occurs. Carpet in control buildings can be a reservoir of fungal spores from normal deposition, including foot traffic.

MATERIALS AND METHODS

The dust samples were collected using 37 millimeter diameter three-piece filter cassettes with polycarbonate filters (pore size of 0.8 microns) and a high volume vacuum pump (Dillon et al. 1996). Generally, sufficient carpet was sampled (vacuumed) to provide a dust sample which filled at least between one-third and one-half of the cassette. The area of carpet surface sampled varied from approximately one to nine square feet. The surface of the carpet was thoroughly vacuumed in multiple directions to pick up as much of the available dust as could be practically collected. The intention being to collect dust from the carpet backing material upwards, spores in any underlayment are not likely to be collected using this method, though conidia that have gown through the carpet backing these would be collected

Samples were analyzed for culturable fungi at three independent microbial laboratories located in the USA. The dust samples were weighed, extracted in sterile water, diluted serially, and inoculated onto culture plates. Plates were incubated at 25 degrees Celsius. The culture plate which produced the clearest growth and colony definition was selected for analysis. All 243 samples were cultured on to Malt Extract Agar (MEA) as a general screening medium. Additionally, 216 of the 243 samples were also cultured on a second agar. The second agar selected was either Dichloran Glycerol agar (DG18), MEA with 20% sucrose (MEA+S), Czapek-Cellulose agar (CCA), or Corn Meal Agar (CMA). The second agar was selected based on the suspected cause of the microbial contamination. Where high humidity was considered to be a potential cause of the contamination and/or xerophilic fungi were expected to be found, DG18 or MEA+S was selected. Where water damage was thought to have occurred and/or where *Stachybotrys chartarum* was suspected as a major contaminant, CCA or CMA was selected (Yang 1995).

Fungal colonies which developed were speciated and enumerated with the total concentration of fungi being calculated and reported as colony forming units per gram of sample. The prevalence of each taxa identified was reported both as raw counts and as a percentage of the total sample. Species identification was based on characteristic morphology and biochemical reaction.

RESULTS

Total Fungi Concentrations

A total of 224 samples were collected in problem buildings. The total fungi concentrations in the 421 cultures of the 224 samples from problem buildings ranged from no growth (less than [<] 400 colony forming units per gram [cfu/g] of sample) to greater than 100,000,000 cfu/g, the highest level to which the laboratory counted. The average concentration of fungi in the problem buildings was 1,844,000 cfu/g with a standard deviation of 8,146,000. The median concentration was 205,882 cfu/g.

A total of 19 samples were collected in control buildings. The total fungi concentrations in the 38 cultures of the 19 samples from control buildings ranged from no growth (<400 cfu/g) to 100,000 cfu/g. The average concentration was 30,256 cfu/g with a standard deviation of 25,317. The median concentration was 23,349 cfu/g.

Distribution of the results by number of culture plates with total fungal concentrations within ranges of concentrations in problem and control buildings is presented in Table I.

TABLE I: Distribution of results by ranges of total fungal concentrations.

	Number of Culture Plates	
Range of Concentrations (cfu/g)	**Problem Buildings**	**Control Buildings**
$> 10^7$	10	0
$> 10^6$ to $<10^7$	80	0
$> 10^5$ to $<10^6$	178	1
$> 10^4$ to $<10^5$	134	28
$> 10^3$ to $<10^4$	15	1
$> 10^2$ to $<10^3$	2	1
> 10 to $<10^2$	0	0
> 0 to <10	2	7
Totals	421	38

Number of Taxa Per Sample.

The average number of identified taxa per culture plate for problem buildings (7.6 for n=421) was similar to the average number for control buildings (7.1 for n=38). The averages were also similar when culture plates with no growth reported where excluded (problem buildings average of 7.6 for n=419 and control buildings average of 8.7 for n=31). The highest number of taxa identified in problem building samples was 17; for control buildings the highest number was 13.

The highest average number of taxa per sample in both problem and control buildings was found in the range of total concentrations between 10^4 to 10^5 cfu/g. However, while most of the control building results were in this range; sample size outside this range is too small to draw accurate conclusions on average taxa per plate. For problem building samples, the average number of taxa decreased with (1) an increase in the total concentration above 10^5, and (2) a decrease in total concentration below 10^4 (Table II).

TABLE II: Average number of taxa by ranges of total fungal concentrations.

	Average Number of Taxa per Culture Plates	
Range of Concentrations (cfu/g)	**Problem Buildings**	**Control Buildings**
$> 10^7$	4.6 (n=10)	NA
$> 10^6$ to $<10^7$	6.6 (n=80)	NA
$> 10^5$ to $<10^6$	7.8 (n=178)	8.0 (n=1)
$> 10^4$ to $<10^5$	8.5 (n=134)	9.2 (n=28)
$> 10^3$ to $<10^4$	5.3 (n=15)	3.0 (n=1)
$> 10^2$ to $<10^3$	1.0 (n=2)	1.0 (n=1)

MAJOR AND DOMINANT TAXA

For this study, a dominant taxa in a sample (culture plate) was defined as constituting greater than fifty percent of the total fungal concentration. 210 of all culture plates contained a dominant taxa. In the problem buildings, 48 percent of the plates contained a dominant taxa compared to 21 percent of the plates from the control building samples.

The most commonly detected dominant species in problem buildings was *Penicillium*, followed by *Cladosporium*, and then *Aspergillus* (Table III). Other species which were dominant in more than one percent of problem building samples included yeasts, *Rhodotorula* (a yeast), *Acremonium*, and non-sporulating fungi.

In the control buildings, the most frequently detected dominant species was *Cladosporium*, followed to a lesser extent by equal percentages of *Penicillium*, *Phoma*, yeasts, and *Epicoccum nigrum* (Table III). *Aspergillus* was never a dominant species in the control buildings. Due to the smaller number of samples from control buildings the dominance of a species in a single sample significantly skewed the results.

For this study, major taxa in a sample was defined as a taxa which constituted 20 to 50 percent of the total concentration. Similar to the dominant taxa, the major taxa in problem building samples were, in descending order, *Penicillium*, *Cladosporium*, and *Aspergillus* species, followed by yeasts (including *Rhodotorula*). In control buildings, the major taxa was *Cladosporium*, again followed to a lesser extent by *Penicillium*, *Phoma*, yeasts and *Epicoccum nigrum*. Similar to the dominant taxa findings, *Aspergillus* species were not identified as a major taxa in the control buildings, although they were reported in 42 percent of the control culture plates (Table III).

TABLE III: Selected fungal taxa present, major, or dominant in culture plates.

	Percentage of Culture Plates					
	Problem Buildings			Control Buildings		
Taxa	Present	Major	Dominant	Present	Major	Dominant
Penicillium spp	86	31	13	63	11	3
Cladosporium spp	80	11	30	76	45	11
Aspergillus spp	70	18	7	42	0	0
yeasts	50	10	4	37	8	3
Rhodotorula	42	6	2	39	5	0
non-sporulating	45	3	1	45	3	0
Epicoccum nigrum	32	3	0	37	8	3
Phoma	28	2	1	58	11	3
S. chartarum	9	2	1	5	0	0

Stachybotrys chartarum was identified in two control culture plates as a minor taxa, and was also identified in nine problem building culture plates, as a major taxa in two, a dominant taxa in one, and as a minor taxa in six other plates.

ASPERGILLUS SPECIATION

In many of the samples, the laboratories were able to identify *Aspergillus* to species level. *Aspergillus versicolor* was the most common *Aspergillus* species identified as present, major, and dominant in the problem building culture plates. *Aspergillus niger* and *Aspergillus sydowii* followed *Aspergillus versicolor* as the most common *Aspergillus* species present in the problem samples. *Aspergillus niger, Aspergillus ustus,* and *Aspergillus fumigatus* were the most common *Aspergillus* species present in control buildings. These species were identified at less than 20 percent of total in the control samples. *Aspergillus versicolor* was identified in only one control sample, as a minor taxa. Table IV presents the percentage of culture plates in both problem and control buildings where selected *Aspergillus* species were identified.

TABLE IV: Selected *Aspergillus* species present, major, or dominant in culture plates.

	Percentage of Culture Plates					
	Problem Buildings			Control Buildings		
Taxa	Present	Major	Dominant	Present	Major	Dominant
A. flavus	4	0	0	3	0	0
A. fumigatus	8	1	0	13	0	0
A. glaucus	1	0	0	0	0	0
A. nidulans	5	0	0	0	0	0
A. niger	22	2	0	18	0	0
A. ochraceus	10	<1	0	0	0	0
A. sydowii	18	1	1	0	0	0
A. ustus	10	1	0	13	0	0
A. versicolor	43	6	4	3	0	0
Other *Aspergillus* spp	9	1	0	3	0	0

Although different media were used to culture the samples, analysis of the results revealed that the type of media used did not affect the *Aspergillus* species concentrations. For samples plat-

ed on two media, similar concentrations for *Aspergillus* species was reported on both the MEA and the second plate used (DG18, CCA, or CMA).

Regional Variations in Problem Building Samples

A total of 112 culture plates were from samples collected in the Northeast USA (Connecticut, New Jersey, New York, Pennsylvania and Massachusetts); 185 were from the Southeast USA (Florida, Georgia, and North Carolina); and 124 were from the Pacific Northwest USA (Oregon and Washington). A slight difference in total concentrations and taxa was noted when results from problem buildings in each region were compared. Table V presents the average, standard deviation, and maximum total fungal concentration by region.

TABLE V: Average and Maximum Fungal Concentrations by Region.

		Total Fungal Concentration (cfu/g)		
Region	**n**	**Average**	**Standard Deviation**	**Maximum**
Northeast	112	1,875,447	8,229,368	61,654,140
Southeast	185	2,099,953	8,944,451	100,000,000
Northwest	124	1,375,189	6,589,863	52,000,000

Generally, the range of fungal taxa identified in samples from all three regions was similar. Certain taxa, however, were more likely or less likely to be present in samples in one region when compared to the other regions. *Aspergillus* species and *Cladosporium* species were more likely to be present, and also more likely to be a major or dominant taxa, in the Southeast. *Curvularia* and *Fusarium* were more likely to be present in Southeast samples. Non-sporulating fungi were less likely to be found in Southeast samples. *Acremonium*, *Phoma*, and yeasts, including *Rhodotorula*, were more likely to be identified in Northwest samples. *Penicillium* was more likely to be a dominant species in Northeast samples. *Cladosporium* and *Stachybotrys chartarum* were less likely to be present in Northeast samples when compared to the other two regions.

DISCUSSION

The analytical results of carpet dust samples suggest that total fungal concentrations greater than 10^5 cfu/g, and certainly those greater than 10^6 cfu/g, will most likely be associated with buildings contaminated with fungi. However, total concentration alone is not always sufficient, because many samples from contaminated buildings contained less than 10^5 cfu/g. The taxa present, especially as dominant or major, can also be an indicator of fungal contamination, especially when considering regional variations. *Penicillium* spp and *Aspergillus* spp, especially *A. versicolor*, when present as major or dominant taxa, are generally associated with problem buildings. In general, control samples do not contain a dominant taxa when three or more taxa are present, unless the taxa is a phylloplane species such as *Cladosporium* or *Alternaria*.

Seven control building culture plates and two problem building culture plates did not contain fungal growth. This is unusual, given the ubiquitous nature of fungal spores. However, some control samples were from new carpet and, in two cases, the samples were collected after the application of biocidal treatment to the carpets.

CONCLUSION

When assessing the results for culturable fungi in carpet dust samples it is important to consider all of the relevant data: total concentration, number of different taxa detected, range of taxa detected, major or dominant taxa present, weight of dust collected, and raw counts of fungal colonies. The results should also consider the geographical region where the samples were collected. Careful consideration should be given to all of these factors in determining the condition of a building from carpet dust samples. Other factors to be assessed include the number of samples collected, whether they represent typical areas, and if appropriate comparison control samples were collected. Finally, dust samples are only a part of a microbial investigation strategy, which should also include visual inspections supplemented by other applicable sampling methods.

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DETECTION AND DECONTAMINATION OF A FACILITY CONTAMINATED WITH FUNGI INCLUDING *STACHYBOTRYS CHARTARUM*: A CASE STUDY

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ABSTRACT

A facility indoor environmental quality investigation was initiated in 1996 due to onset of employees' health symptoms after fifteen minutes exposure in the facility. The investigation was to determine the extent of contamination, identify the causative agents, design decontamination specifications, and develop and implement a monitoring strategy for clearance sampling.

Massive fungal growth was observed on various surfaces. Many types of microbiological samples were collected throughout this two-story facility. Very high fungal levels were detected on bulk and wipe samples (10^7 CFU/g and 10^6 CFU/in^2). Three rounds of air samplings were conducted. Outdoor fungal levels were at 10^2 CFU/m^3 with *Cladosporium* as the predominant fungi. However, indoor fungal levels were consistently at least 10^4 CFU/m^3. Indoor fungal flora was dominated by *Aspergillus*, *Penicillium*, and *Stachybotrys chartarum*. Analytical results showed that basement, ground floor, and ductwork were contaminated.

Specifications were developed to decontaminate the facility and the ductwork. Ten percent (10%) bleach solution and Oxine® brand of chlorine dioxide (500 ppm) was used as the disinfectant for the facility and the ductwork, respectively. Final clearance samples were taken to evaluate the effectiveness of the cleaning. Air sampling results showed that airborne fungal levels in the facility were compatible to those of outdoors (230 and 224 CFU/m^3, respectively), with a similar fungal flora. Results from final clearance surface samples, taken after completion of second ductwork cleaning, showed that surface fungal burden of this facility was not different from that of a reference building.

KEY WORDS

Stachybotrys, *Penicillium*, *Aspergillus*, indoor air quality, bioaerosol, fungi, decontamination, duct cleaning.

INTRODUCTION

A two-story recreational pavilion was vacant for more than six months. Employees entered the basement of this facility to perform cleaning. Within fifteen minutes of exposure, the cleaning crew was forced to rush outdoors due to the onset of health symptoms. They experienced dif-

ficulty in breathing, chest pain, headaches, and some vomiting. The US Public Health Service, Federal Occupational Health (FOH), was requested by the agency to conduct an indoor environmental quality investigation to determine the cause and extent of contamination, identify the causative agents, design decontamination specifications, and develop and implement a monitoring strategy for clearance sampling.

The basement consisted of several rooms with various functions: offices, classrooms, mechanical rooms, kitchen, rest rooms, and storage rooms. The first floor contained a storage room, kitchen, rest rooms, and an office. All areas had floor tile coverings except for the classrooms and offices where carpet was installed.

MATERIALS AND METHODS

Microbiological Sampling

Various microbiological samples were collected; namely, air, surface wipes, contact plates, and bulk samples. Two percent malt extract agar (MEA) (Hunter et al. 1980, Miller et al. 1988) and cellulose Czapek agar (CCA) (Park, 1973) were used for recovery of general fungi, and cellulose-loving fungi, respectively.

Air samples were collected, at about 3 - 5 foot above the floor, using Andersen® N-6 samplers at a flow rate of 28.3L/min. Samplers were surface-disinfected using alcohol wipes before each sampling.

Wipe samples were collected using sterile cotton swabs (BBL® Aerobic Transport System) to wipe various surface areas of 1 square inch. Rodac® contact plates (65-mm in diameter) filled with 2% MEA or CCA were used for contact plate sampling. Samples were taken by placing the agar-filled contact plate against the selected hard surface for about 5 seconds.

Bulk samples, such as drywall, fiberglass insulation materials, and ceiling tiles were collected from the facility and each was put into a gallon-size Zip-lock® bag with a proper label. Tools used for cutting were surface-disinfected with 70% isopropyl alcohol before each sampling.

Laboratory Analyses

All samples collected from the facility were mailed to the laboratory for next morning delivery. Wipe and bulk samples were processed upon receipt of the shipment.

Wipe samples were suspended in sterile distilled water, diluted serially, and inoculated onto MEA and CCA plates. Bulk samples were weighed, suspended in sterile distilled water, and followed the aforementioned dilution plating. At least three series of dilution were used for each sample.

All plates were incubated in a 25°C incubator. They were examined every other day for up to 2 weeks to assure the full recovery of fungi. Fungal identification was based on colony morphology, spore and conidia formation. The total number of fungal colonies formed on each plate were counted and recorded. Fungal concentrations were presented as colony forming units (CFUs) per measuring unit. For air samples, fungal levels were presented as CFUs per cubic meter of air (CFU/m^3). Similarly, CFU/in^2, CFU/plate, and CFU/g were used for wipe, contact plate, and bulk samples, respectively.

RESULTS AND DISCUSSION

Field Observation

During an initial walk-through, water line and massive fungal growth were observed on gypsum drywall surfaces up to 5-foot high (Figure I). Visible fungal growth was also observed on supply air diffusers, chairs in the classroom, ceiling tiles, and floor tiles. The ventilation systems had been turned off and the failure of sump pumps had caused frequent flooding in the basement during the off season.

FIGURE I. Massive fungal growth on wall surfaces and ceramic tiles at the basement.

Microbiological Results

Very high fungal concentrations were detected from various types of samples. *Aspergillus* and *Penicillium* were the predominant fungal genera detected from indoors on MEA plates. *Stachybotrys chartarum* (SC) was detected from indoor samples on CCA plates, and sometimes on MEA plates, too.

Air Samples

First Round

Quiescent, air samples were collected from outdoors and various locations indoors. One outdoor airborne sample showed fungal concentration of 530 CFU/m^3 with a diverse fungal population. *Cladosporium* was the predominant fungi, followed by *Alternaria*. No SC was detected

from this outdoor sample. Indoor airborne fungal levels were at least 4.6 x 10^4 CFU/m^3 (more than 400 fungal colonies were detected from each MEA plate). *Aspergillus* and *Penicillium* were the predominant genera recovered from MEA plates. *Stachybotrys chartarum* was detected from all nine CCA plates collected indoors, ground floor and basement included, with concentrations ranging from 53 CFU/m^3 to greater than 4.6 x 10^4 CFU/m^3 (Table I).

TABLE I: Airborne fungal levels and fungal populations in the facility and outdoors, cultured on MEA plates at 25°C for 14 days.

Sampling Locations	Total Fungal Levels on MEA (CFU/m^3)	Alt** (%)	Asp (%)	Cla (%)	Pen (%)
Outdoors	530*	20	7	63	7
Indoors	> 4.6 x 10^4	0	56	0	40

* All numbers presented were concentrations calculated through corrected particle counts (Anonymous, 1984).

** Alt: (*Alternaria* level / total fungal level) * 100.
Asp: (total *Aspergillus* levels / total fungal level) * 100.
Cla: (*Cladosporium* level / total fungal level) * 100.
Pen: (*Penicillium* level / total fungal level) * 100.

Second Round

Due to high fungal levels detected from indoor samples collected from the first round, the sampling time for indoor samples was reduced from two minutes to 15 seconds. Sampling was conducted at the basement and ground floor. Results showed that even with a 15-second sampling, all seven samples collected showed at least 400 fungal colonies (fungal levels were greater than 3.4 x 10^5 CFU/m^3) with a similar fungal flora. It is important to note that after five-day incubation at 25°C, storage mites were observed on most of the indoor samples on MEA plates.

Third Round

Another air sampling was conducted to simulate the situation during the onset of employees' health symptoms. Air samples were collected from the outdoors, to the first floor entrance, to the stairwell and then to the basement. Results again confirmed much higher fungal levels indoors than those of outdoors. *Cladosporium* was the predominant fungi outdoors, and no SC was detected. *Penicillium* and *Aspergillus* dominated indoor fungal flora (Table II). Furthermore, SC was detected from every indoor sample collected on CCA plates. As employees entered the building, they were exposed to higher fungal levels as they proceeded from outdoors to the entrance, stairwell, and the basement. Moreover, employees were exposed to higher levels of *Aspergillus* (including *Aspergillus niger* and *Aspergillus versicolor*), *Penicillium*, and SC as they arrived at the basement (Table II).

Outdoor air samples were collected daily (total of 22 samples) during detection and decontamination to detect any daily variation. Results showed that fungal levels ranged from 353 CFU/m^3 to 1,790 CFU/m^3. *Cladosporium* was the predominant fungi throughout this sampling period. Indoor samples were also collected from different areas periodically to monitor the decontamination.

TABLE II: Airborne fungal levels and fungal taxa on samples collected from the facility and outdoors, cultured on MEA plates at 25°C for 14 days.

Sampling Locations	Total Fungal Levels on MEA (CFU/m³)	Asp* (%)	Bas (%)	Cla (%)	Pen (%)	Sc (%)	Tri (%)	PAS (%)
Outdoors	353	0	10	80	0	0	10	0
Ground Floor								
Entrance	1.2×10^4	60	0	18	21	0	0	81
Stairwell	2.1×10^4	65	0	21	11	0	1	76
Basement	$> 4.6 \times 10^4$	79	0	4	13	3	1	95

* Asp: (total *Aspergillus* levels / total fungal level) * 100.
Bas: (Basidiomycetes level / total fungal level) * 100.
Cla: (*Cladosporium* level / total fungal level) * 100.
Pen: (*Penicillium* level / total fungal level) * 100.
SC: (*Stachybotrys chartarum* level / total fungal level) * 100.
Tri: (*Trichoderma* level / total fungal level) * 100.
PAS: Asp + Pen + SC.

Wipe, Contact Plate, and Bulk Samples

Wipe, contact plate, and bulk samples were collected to identify the source of contamination (Dillon et al. 1996). Surface samples (wipes and/or contact plates) were collected from gypsum wallboard surfaces, chair surfaces in the classroom, supply grill, wall cavity, and inside the ductwork. Fungal concentrations ranged from 2.4×10^4 CFU/in² to 1.6×10^7 CFU/in², and 1.4×10^3 CFU/g to greater than 1.6×10^6 CFU/g, for wipes and bulks, respectively. Predominant fungal genera recovered from these samples on MEA plates were *Penicillium* and *Aspergillus*. *Stachybotrys chartarum* was also detected on CCA plates from various samples, such as: ceiling tiles, gypsum wall board, wall surfaces, insulation materials in the walk-in refrigeration units, and inside wall cavities. Examples of fungal concentrations in these materials are presented in Table III.

TABLE III: Fungal concentrations and taxa of bulk samples collected from this facility and cultured on MEA plates at 25°C for 14 days.

Bulk Materials	Fungal Concentrations (CFU/g)	Fungal Taxa on MEA
A ceiling tile	8.5×10^5	*Stachybotrys chartarum* (66 %)
		Aspergillus (27 %)
		Penicillium (7 %)
A gypsum wallboard	1.1×10^6	*Penicillium* (80 %)
		Stachybotrys chartarum (10 %)
		Cladosporium (8 %)
		Aspergillus (1 %)
		Pithomyces (1 %)

These results confirmed visual observation and identified sources of heavy fungal contamination in the facility, ductwork included, both in the basement and on the ground floor. *Aspergillus*, *Penicillium*, and *Stachybotrys* were the fungal contaminants.

DECONTAMINATION

A large-scale remediation program based on New York City guideline (New York City Department of Health, 1993) was developed by the FOH and conducted in the facility by a contact company. Both basement and ground floor were contained with negative air pressure. The decontamination began with the ground floor, which was a less contaminated area. The specifications included containment guideline, personnel requirements, waste disposal, clean-up, personal protection equipment, project monitoring, and final project clearance criteria (Table IV). Contaminated bulk materials, confirmed by visual observation and/or results from fungal analyses, such as: gypsum wallboards, ceramic tiles, wooden studs, ceiling tiles, carpet, fireproofing and fiberglass insulation materials, were removed and disposed of. All surfaces were high efficiency particulate air (HEPA) vacuumed followed by wet-wiped with a 10% bleach solution. Due to State, where the facility resides, regulation, bleach solution was not allowed to apply on the ductwork surfaces of the heating, ventilation, and air-conditioning (HVAC) system. Thus, the ductwork surfaces of the HVAC system were HEPA-vacuumed followed by the application of Oxine® brand of chlorine dioxide at 500 parts per million (ppm), the only biocide approved by the State.

TABLE IV: Final project clearance criteria used for decontamination of this facility.

SEQUENCE	CONTENTS
1	A visual inspection will be conducted to ensure no visible fungal growth on any surfaces
2	A combination of airborne and surface contact plate and/or wipe samples will be col lected and laboratory analyzed prior to removal of containment barriers.
3	Indoor airborne fungal levels will not be greater than the average outdoor fungal level before barriers are removed. Fungi recovered indoors shall be similar to those of out doors.
4	*Stachybotrys chartarum* (SC) shall not be detected in any clearance samples. Contractors will be required to re-clean areas until all SC fungi are at non-detectable levels before barrier removal.
5	Results from wipe/contact plate samples shall not be greater than the average of those taken from representative reference areas.

CLEARANCE

Air, contact plates, and wipe samples were collected during decontamination to monitor the decontamination efforts. Final clearance samples were collected from the facility after at least 48 hours of final clean-up, to evaluate the effectiveness of the overall cleaning.

Mean airborne fungal levels in the facility were not different from those of outdoors (230 CFU/m^3 vs. 224 CFU/m^3, p= 0.844). Moreover, diverse fungal populations were detected from both indoor and outdoor samples. No SC was detected from any air samples.

Contact plate and wipe samples were collected from various surfaces during clearance sampling. Results indicated that SC was still present on surfaces of ductwork. The surfaces of duct-

work were then cleaned once again with Oxine®. Results from final surface sampling, after completion of second ductwork cleaning, indicated that fungal levels on surfaces of this facility were not significantly different from those of the adjacent, reference building (Table V).

TABLE V: Mean fungal levels from surface samples collected during final clearance sampling.

Sample Types	The Facility	Reference Building	Significant Level
Contact Plates (CFU/plate)	29 + 7	18 ± 5	$p = 0.321$
Wipes (CFU/in^2)	30 ± 17	56 ± 40	$p = 0.496$

DISCLAIMERS

The views expressed in this document are those of the author and do not represent the official Federal Occupational Health (FOH), United States Public Health Service policy. Mention of company names or products does not constitute endorsement by the author or the FOH.

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CASE STUDY: AIRBORNE CONCENTRATIONS OF TRICHODERMA AND STACHYBOTRYS LINKED TO MYCOTOXICOSIS

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ABSTRACT

The affected individual developed symptoms approximately 55 days after exposure to a working environment containing significant fungal contamination. Initial symptoms included bronchitis, swelling, spastic colon, severe headaches, and fatigue. Later, abdominal pain, nausea, diarrhea, and loose teeth were reported. Three physicians having specialties in internal medicine, toxicology, and neurology independently diagnosed exposure to an "unknown" environmental toxin; however, the physicians were not provided with environmental results from the work place. The work environment indicated airborne concentrations of *Trichoderma viride* and *Stachybotrys chartarum* at 494 CFU/m^3 and 212 CFU/m^3, respectively. Active *Trichoderma viride* and *Stachybotrys chartarum* growth sites were documented at levels of 3.3 x 10^4 CFU/g and 2.0 x 10^7 CFU/g; respectively. Although these contaminants were exclusive to the individuals work area, the CIH/CSP represented the data as "typical" for indoor environments. As a result, no exposure data was provided to the attending physicians. After 5 months of exposure the individual became too ill to return to work. Within 1 month of removal the symptoms begin to subside. Complete symptom cessation resulted after 12 months. The absence of specific human dose-exposure data relative to the various mycotoxins produced by *Trichoderma viride* and *Stachybotrys chartarum* does not support a valid medical claim relative to an aerosol-induced mycotoxicosis. However, anecdotal, circumstantial, and environmental information strongly supports the potential for this event to have occurred. This study demonstrates the current void that exists relative to both the knowledge and availability of mycotoxin diagnostic methods in the medical field and suggests the immediate need for education, training, and research relative to aerosol generated mycotoxicoses.

KEY WORDS

Stachybotrys chartarum, Trichoderma viride, mycotoxins, mycotoxicosis, airborne concentrations.

INTRODUCTION

The fundamental nature of the exposure to an environmental antagonistic agent and it's ability to manifest as an environmental disease often eludes the identification of traditional medical cause and effect relationships (Burge, Harriet, 1993; Davidsohn et al.; Davis et al. 1968; Ganong, William; Pope et al. 1993; Walsh et al. 1989; Wilson, 1974; Brief,. Bernath. 1988;. Burge,1995; Cox, Wathes, 1995). The ability to clearly evaluate essential causal information is

complicated to an exponential degree by numerous variables associated with individual antagonistic agents, environmental conditions, and host susceptibility (Hansen, 1991; Jensen, Wright; Kwon-Chung et al.; Larone;). The objective of this investigation is to improve the understanding of airborne induced mycotoxicoses in humans. Moral, ethical and legal considerations prohibit the use of humans in research involving airborne exposure to fungal bio-particulate and mycotoxins. Exposure data from contaminated indoor environments may offer one of the few sources available to evaluate the effect of airborne components and the ability to induced mycotoxicoses in humans.

While often considered to be outside of traditional, medical cause and effect relationships, the diagnoses of environmental disease often requires, at least in part, the use of specific elements of human behavior. For example, even a subtle, pre-knowledge of a potential environmental disorder has, under certain circumstances, has been demonstrated as capable of inducing psychogenic manifestation of disease. The lack of any "pre-existing" knowledge about a specific disease would, in theory, eliminates the potential for psychogenic interference in true disease expression. Therefore, the presence of symptoms in uninformed individuals offers a unique condition to evaluate specific relationships of environmentally induced disease.

The development of symptoms often results in an individuals accelerated desire to determine causation. Accelerated efforts may result in misdiagnoses, mistreatment, and changing environments, which ultimately interfere with the actual identification of a specific environmental cause and effect event. Therefore, only in a few circumstances, does a critical window of opportunity exists to observe specific events involving environmental exposure and symptomatology. Historical data about the symptomatic individual is often critical for the exclusion of extraneous data that can bias diagnoses.

The individual was a 45-year-old white female, married, with has one healthy daughter. She rarely drank alcohol and did not use tobacco products. She was a life-long career educator with a history of exemplary performance with local, state, and national recognition. Recently the President of the United States had formally recognized the individual as an Outstanding Educator at the White House. In 1993 she was promoted to Dean of a "new" innovative High School which specialized in advanced math and science instruction. The "new" school was actually a "remodel" hospital, which had been abandoned since 1986. Serious roof leaks occurred during the period of non-occupancy. The school district began to repair the building, however; budgetary shortcomings prevented to total repair of the roof and mold contaminated interior space. The subject in this case was officed in a location that was contiguous with an area having "large quantities of visible, dark mold growing on the wall". A common heating, ventilation, and air conditioning (HVAC) system also existed between these locations. No other offices in the space demonstrated a similar proximity with respect to the contaminated area. The affected individual developed symptoms approximately 55 days after exposure to this environment.

METHODOLOGY

MEDICAL

Initial symptoms included bronchitis, swelling, spastic colon, severe headaches, and fatigue. Later, abdominal pains, nausea, diarrhea, along with loose teeth were reported. Symptoms were continuous from August 1993 through December 1995 until the individual remained too ill to return to work. Three physicians practicing internal medicine, toxicology, and neurology, respectively and one dentists provided medical diagnoses of the affected individual. Patient diagnoses and testing included: physical examination, contrast and non-contrast magnetic resonance imaging (MRI), hematology, serology, neuroexamination, and extensive blood chemistry. "Texas Workman's Compensation" claim was filed as a result of the patients inability to return to work.

ENVIRONMENTAL

In response to the filing of a Workman's Comp. Claim in December 1995, a Certified Safety Professional/Certified Industrial Hygienist (CSP/CIH) was hired by the school district to evaluated the work environment. Fungal bioaerosols were evaluated utilizing an Andersen N6 sampler with malt-yeast extract agar plates (Robertson, 1995;.Morey et al. 1990; Etkin, Schmidt. 1994; Atlas; ACGIH, 1989). Bulk samples of suspected contaminates were also collected, serial diluted and aseptically transferred to malt-yeast extract culture plates. Individual fungi were identified using standard and accepted mycological techniques (O'Leary, 1989; Jensen, Wright; Alexopoulos; Kindrick, 1985; Barnett, Hunter, 1972; Smith, Grant, 1984;

RESULTS AND DISCUSSION

The three physicians treating the individual independently diagnosed exposure to an "unknown" environmental toxin(s). The environment indicated airborne concentrations of *Trichoderma viride* and *Stachybotrys chartarum* at 494 CFU/m^3 and 212 CFU/m^3, respectively. Active *Trichoderma viride* and *Stachybotrys chartarum* growth sites were documented at levels of 3.3 x 10^4 CFU/g and 2.0 x 10^7 CFU/g; respectively. *Trichoderma viride* and *Stachybotrys chartarum* were not observed in samples collected at other indoor locations of the same school, outdoors, or at other buildings utilized for control. Despite the presence of active, indigenous growth and documented abnormal exposure, the CSP/CIH project manager represented the data as "normal" and "typical" for indoor exposure (Morey, Jenkins., 1989; Yang et al. 1993;. Toth, 1992; Pasanen et al. 1990;. Reponen et al. 1990;. Reynolds et al. 1990;. Miller et al. 1998;.Holt, 1993;). As a result, these data were not forward to the physicians tending this case. Within 1 month of removal from the environment the symptoms begin to diminish. Complete symptom cessation resulted after 12 months.

CONCLUSION

In this case, the absence of specific human dose-exposure data relative to the various mycotoxins produced by *Trichoderma viride* and *Stachybotrys chartarum* does not support a valid medical

claim relative to an aerosol-induced mycotoxicosis. However, anecdotal, circumstantial, and environmental information strongly supports the potential for this event to have occurred. The airborne quantification of prominent toxigenic fungi at a time that is consistent with specific diagnosed exposure to environmental toxin(s), are extremely rare. Therefore, airborne data collected under these specific circumstances may be useful in improving the evaluation of indoor environments and the potential for mycotoxicosis. The author concludes that chronic, airborne exposures to the concentrations of *Trichoderma viride* and *Stachybotrys chartarum* observed in this case should be considered as potentially hazardous and warrants further investigation as to a mycotoxigenic environment.

ACKNOWLEDGMENTS:

This paper would like to acknowledge Eileen Beck of Bryan/College Station, Texas. Her unwarranted exposure to this hazardous environment resulted in long-term medical suffering, forced an early retirement, and cast skepticism on a previously unblemished and exemplary professional career as a public educator. In addition to a hazardous environmental exposure, Ms. Beck also fell victim to the self-serving actions of a political system which had the power to influence certified safety professionals, industrial hygienists, and others in the misrepresentation of her environmental exposure, care, and treatment. I wish to formally recognize Ms. Beck's extended tribulations in this incident and thank her for her unique contributions in the emerging field of environmentally induced mycotoxicoses.

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FUNGAL GENERA IDENTIFIED FROM FLOODED WALL CAVITIES

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ABSTRACT

Field investigators are often confronted with the situation of evaluating whether or not fungal amplification is present on the back side of drywall in walls which have sustained a moisture intrusion. When there is no visible source of fungal growth in a room with higher than expected levels of culturable fungi and a pathway exists for migration of fungal spores into the room, growth on the interior of the wall cavity must be considered. This study was conducted to assess if culturable air sampling of wall cavities is a valuable tool to assist in making this determination.

The fungal genera present in the wall cavities were determined by collecting air samples through a hole in the wall with an Anderson N6 stage impaction sampler on selective media with laboratory analysis. The goal of the study was to determine the fungal genera present in the inside of the wall cavities which have sustained a moisture intrusion (flood) and compare them to the fungal genera in wall cavities which have not sustained a water intrusion (non flooded).

The analysis results showed an increase in the total colony counts and a significant shift from *Cladosporium* species to *Aspergillus/Penicillium* species in the wall cavities which had sustained the water intrusion. Statistically, in the flooded wall cavities, the sum of *Aspergillus* and *Penicillium* is higher by more than three times the standard deviation.

KEY WORDS:

water damaged walls, water intrusion, drywall, stud bay, fungal genera, *Aspergillus, Penicillium, Cladosporium.*

INTRODUCTION

Two separate walls with 2 stud bays each were constructed in an interior space. One wall was flooded with clean potable water in a manner such as frequently occurs during a small flood in a residential building. The flooded wall was air dried without blowers or dehumidifiers. The second wall cavity was used as a control. Culturable air samples were collected from all wall cavities prior to flooding to serve as controls. After flooding, air samples were collected from the two water damaged wall cavities, the two control wall cavities and the ambient air.

METHODS AND MATERIALS

Wall Construction and Flooding:

Two sets of walls were constructed with commercially available lumber (2x4's) and 1/2 inch gypsum wallboard (drywall). Each wall containing two stud bays to duplicate as close as possible the construction of wall cavities in a residential home. A sealing coat, texture coat and final flat latex paint coat were applied to the walls by a professional painter. The sides, front and back, where the drywall met the 2x4 framing members, were sealed with a latex caulk. The top and bottom edges were not sealed.

1.5 inch circular holes were cut at the bottom of each stud bay to facilitate use of the Andersen N6 impaction sampler to collect air samples from the cavities and for insertion of the *Dickson THDx* probe for temperature and humidity monitoring. The holes were plugged with rubber stoppers when not being used for sampling or recording temperature and humidity.

A basin was formed with plastic sheeting material under one of the walls. Clean water was introduced into the basin to a height of 1 inch and allowed to wick into the material. Ambient and wall cavity temperature and humidity were monitored from all stud bays with strip chart recorders. Wicking of the water by capillary action was also measured. The water was removed from the basin after six hours.

Culturable Microbial Air Sampling:

The Andersen N6 impaction sampler was used to collect duplicate samples on MEA (Malt Extract Agar) and DG18 (Dichloran glycerol) media supplied by *P&K Microbiology Services, Inc.* Sampling was conducted for one minute at a flow rate of 28.3 liter per minute. The results are expressed in colony forming units (CFU) per cubic meter of air (m^3). The samples were collected in each of the four stud bays by placing the orifice of the Andersen sampler into the hole at the base of the cavity. The walls were tapped 5 times with the palm of the hand prior to sampling. Ambient air sampling was conducted in the room. Baseline sampling from all stud bays was conducted three times in a three month period prior to the flooding. Cavity air sampling was conducted at four different times starting two months after flooding occurred and the materials were dry. The non-flooded wall was used as a control.

Primary Air Flow Calibration: The air sampling pump was calibrated with a *Bios* DryCal DC-1 calibrator prior to conducting each set of samples. The *Bios* DryCal DC-1, is considered a primary standard. The accuracy of the instrument is at a range of $\pm$ 2 % at a single reading and $\pm$ 1 % at averaged readings.

Temperature and Relative Humidity:

A *Dickson* TH550 hand held Digital Thermo-hygrometer was used to record temperature and humidity levels. A *Dickson* THDx circular chart recorder was used for continuous monitoring in flooded and non flooded wall cavities, until temperature and humidity returned to ambient levels. The humidity accuracy of the two instruments is $\pm$2% between 10 and 90% RH with a range 0 to 95%RH. The temperature range of the instruments is -20 to +120 degrees Fahrenheit with a temperature accuracy of $\pm$1.8 degree Fahrenheit.

Moisture Measurements:

The Delmhorst BD-8 was used to record the moisture levels in the walls. The Delmhorst BD-8 is a portable, battery operated instrument designed to give relative qualitative moisture readings in plaster, drywall, brick, concrete, lumber and roofing materials. It incorporates semi conductor technology and has an internal calibration and a battery check.

RESULTS

After all of the bulk water was removed from the wall, the capillary action continued to wick moisture up the drywall for another 18 hours. 100% moisture readings reached a maximum height of 12 inches above the floor level under the given flooding conditions. The flooded wall air dried out in about 2 weeks. There were no visible signs of water intrusion (staining) on the wall and no visible fungal growth.

TABLE I: Range of Fungal Counts in Air Samples Collected.

Location	Wall Cavity #	MEA* # of plates	DG18* # of plates	MEACFU/m³* Spread (range)	DG18 CFU/m³ Spread (range)
Ambient Air		11	9	247-4,981	212-4,240
Non flooded	3	9	7	248-1,732	318-1,413
	4	10	7	317-1,554	353-1,271
	2	3	3	636-1,696	212-741
	1	3	3	423-777	176-741
Flooded	1	7	5	635-4,558	1,130-4,311
	2	6	4	3357-9434	5,336-9,116

* **MEA** Malt Extract Agar, **DG18** Dichloran Glycerol Agar, **CFU/m³** Colony Forming Units/m³

Review tables II, III and IV for a list of all of the fungal genera identified. The *Aspergillus* species listing includes a predominance of *Aspergillus versicolor* and *Aspergillus niger*. It was not a part of this study to identify to species the *Aspergillus* or *Penicillium* cultures.

The increase of total fungal activity is also documented on both media MEA and DG18.

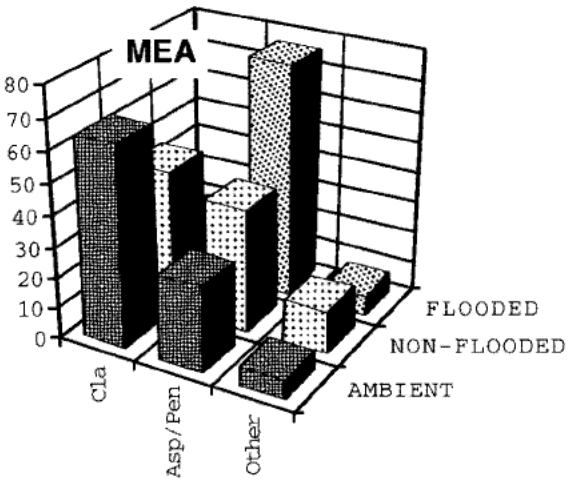


TABLE V: Distribution of Fungal Genera on MEA media.

Genera MEA	Ambient [%]	Non-flooded [%]	Flooded [%]
Cladosporium (Cla)	65 (20)	46 (26)	18 (12)
Aspergillus/Penicillium (Asp/Pen)	28 (18)	40 (23)	76 (12)
Other	6 (6)	14 (12)	6 (6)

(standard deviations for percentage in parentheses)

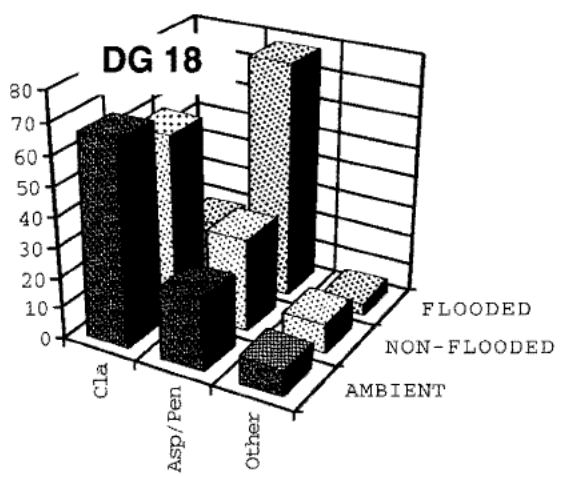


TABLE VI: Distribution of Fungal Genera on DG18 media.

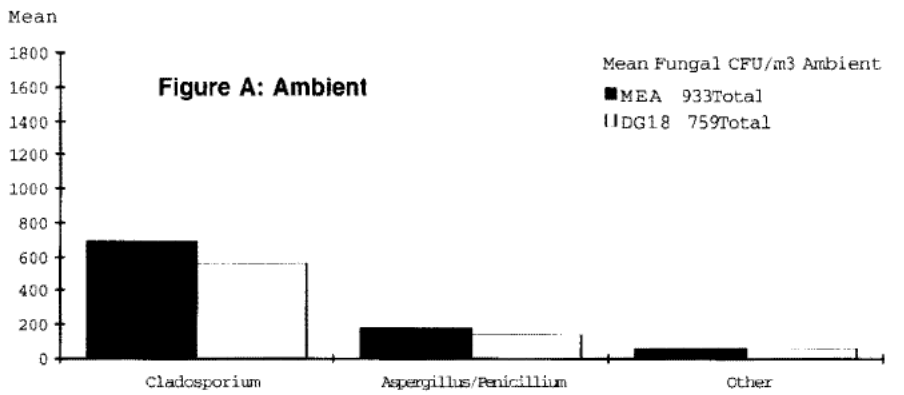
Genera DG18	Ambient [%]	Non-flooded [%]	Flooded [%]
Cladosporium (Cla)	67 (24)	59 (26)	18 (11)
Aspergillus/Penicillium (Asp/Pen)	24 (17)	31 (24)	78 (8)
Other	9 (9)	10 (12)	4 (5)

(standard deviations for percentage in parentheses)

DISCUSSION

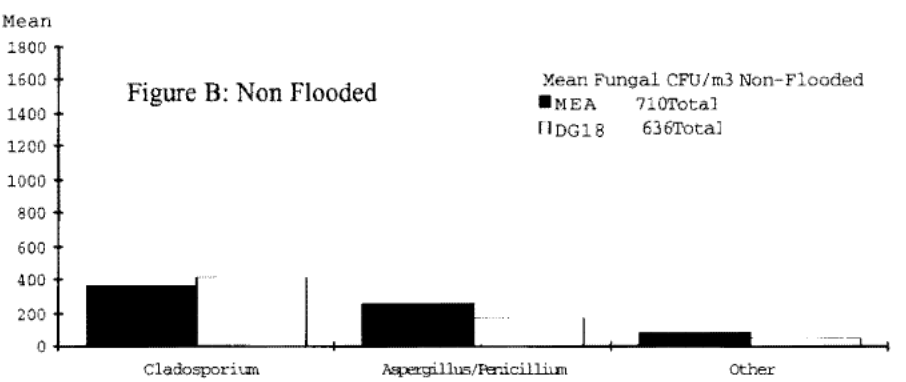
Observance of graphs Figure A, B, & C, Table VII, shows the comparison of total fungal colonies cultured on MEA and DG18 media. It is interesting to note that the total fungal colonies are very similar and the distribution almost the same on the two media.

TABLE VII: Comparison of Mean Concentration on MEA and DG18 media.



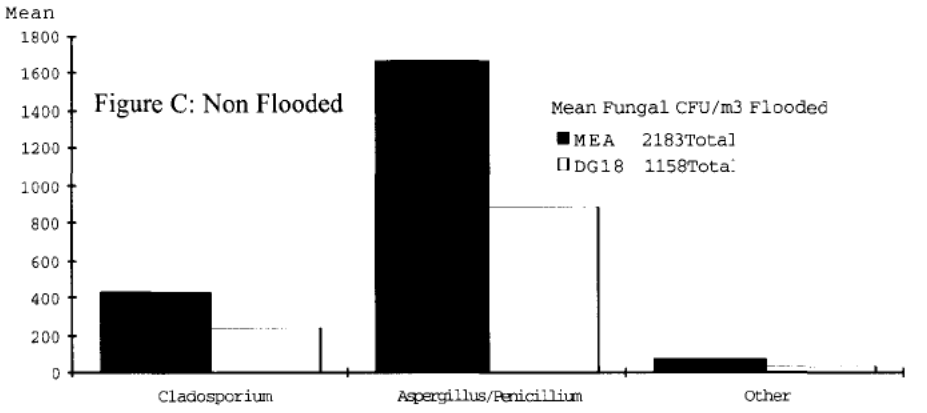
Genus	MEA Media	DG18 Media
Cladosporium	693 (754)	561 (766)
Aspergillus/Penicillium	177 (139)	141 (106)
Other	64 (63)	57 (49)
Total	933 (871)	759 (820)

standard deviations for CFU/m³ in parentheses



Genus	MEA Media	DG18 Media
Cladosporium	367 (366)	411 (316)
Aspergillus/Penicillium	261 (222)	173 (135)
Other	82 (48)	52 (55)
Total	710 (435)	636 (341)

standard deviations for CFU/m³ in parentheses



Genus	MEA Media	DG18 Media
Cladosporium	437 (448)	239 (153)
Aspergillus/Penicillium	1671 (833)	884 (213)
Other	75 (69)	36 (36)
Total	2183 (1039)	1158 (336)

standard deviations for CFU/m^3 in parentheses

CONCLUSIONS

Air samples collected with an Anderson N6 stage culturable air sampler from cavities of walls that have a history of water damage are likely to yield elevated levels of fungi with *Aspergillus/ Penicillium* in the majority. The level of total CFU/m^3 can also be expected to increase in the flooded wall cavities compared to the levels in ambient air samples.

The flooded wall cavities in our study had no visible water stains or fungal growth. This would seem to indicate that drywall walls which have sustained a water intrusion do not have to show water stains or fungal growth on the exterior for the growth to have occurred on the inside.

Additional investigation needs to be conducted to compare samples collected from wall cavities with known water intrusion in actual structures with the ambient air samples and with wall cavities (in the same structure) where there is no known history of water intrusion.

Although identifying colonies to species was not a part of this study, we do feel that it should be done. Casual observation of the culture plates revealed different *Penicillium* in the ambient environment than in the wall cavity samples. Speciation of *Cladosporium*, *Aspergillus* and *Penicillium* would also be helpful to create a data base of fungal species most commonly found in the air of wall cavities which have sustained water damage.

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humidity chart recorders, Hector Figueroa, Felicia Neumann and Gene Tallon for their technical support.

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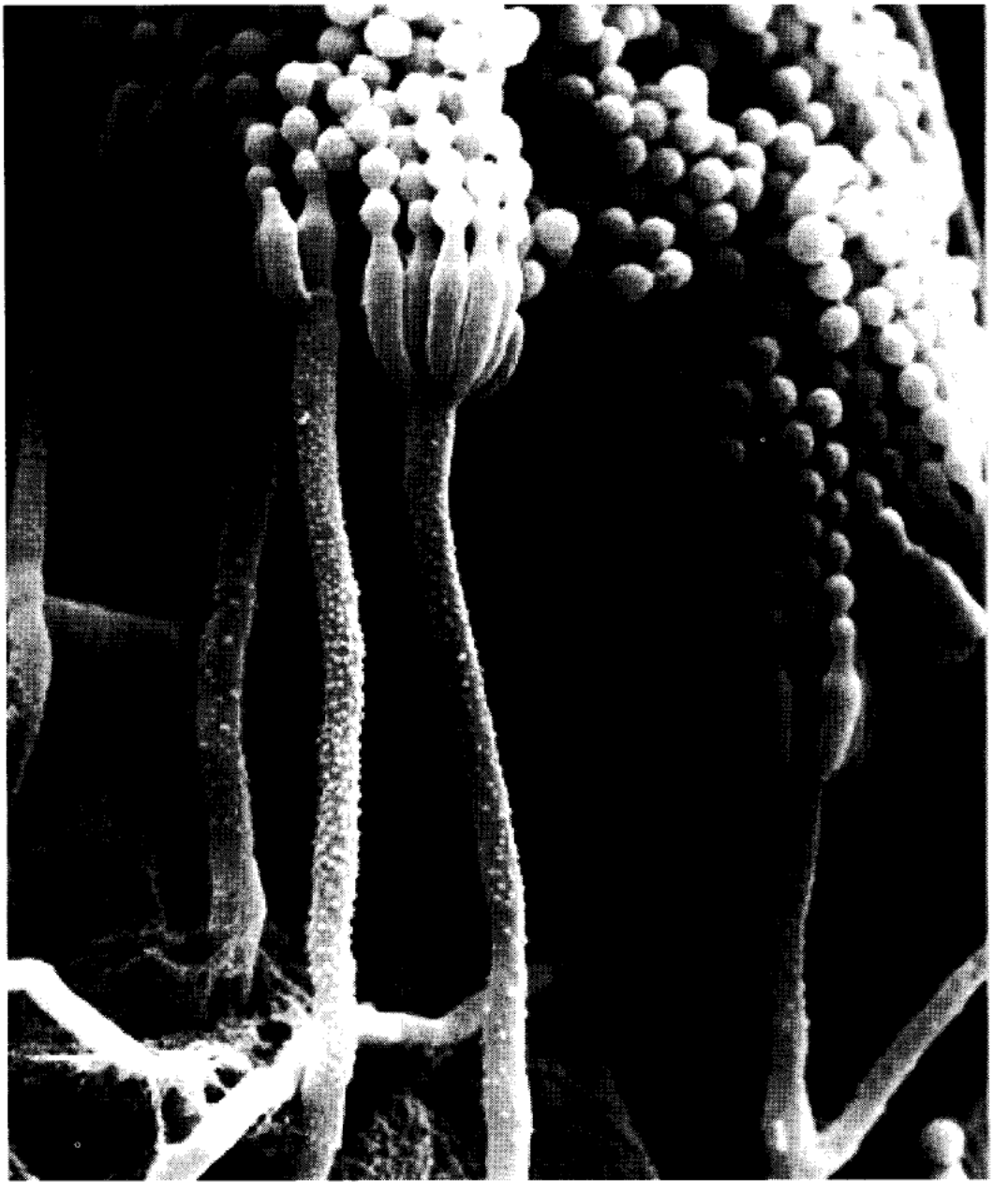

Penicillium glabrum, EM 8,000 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands

Chapter 3

Microbiology

EXPOSURE MEASURES FOR STUDIES OF MOLD AND DAMPNESS AND RESPIRATORY HEALTH

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ABSTRACT

This paper reviews some basic principles underlying the measurement of fungi in air samples and dust to describe their quantitative and qualitative value. We compare the results of such measurements with our experience in the Wallaceburg study wherein ca. 400 homes in southern Ontario were studied for various exposure and health outcomes. Until more powerful methods to determine quantitative and qualitative exposures to fungi are developed, the attributable risk of fungal contamination in residential housing for public health cannot be estimated with accuracy. The most reliable information on mold exposures in residential housing can be obtained by measuring moisture source strength, detailed study of areas of visible mold plus some mycological testing of building materials to determine where the growth is occurring beyond that visible to the naked eye and qualitative and quantitative analysis of molds in settled dust.

KEY WORDS

molds, health, exposure assessment, air samples

INTRODUCTION

The fungal toxin aflatoxin is the most potent chemical carcinogen known. From the time of its initial regulation circa 1971, there has been considerable debate regarding its human carcinogenicity. This was not finally resolved until 1992 when the results of a number of prospective epidemiological studies making use of serum aflatoxin-adduct concentrations as exposure measures became available. Aflatoxin is a complete human carcinogen but importantly affects response to the carcinogenic viruses hepatitis B and C such that there is a 60 fold greater likelihood of getting liver cancer in the presence of both agents (IARC 1993; Quian et al. 1994). Exposure assessments based on bulk commodity analysis or of food on the dinner plate were very unreliable (Hudson et al. 1992). This case illustrates an important point: even when the agent has exceptional potency and clear endpoint (liver cancer), methods that measure true body exposure were required to determine health significance. It further illustrates the need to understand how multiple exposures might confound, interact or potentiate any health response.

Since 1982, ca. 30 studies have been conducted on the association of dampness, mold and respiratory health in residential housing in Europe and North America. A study of 4,600 children

from six cities in the northeast US demonstrated that the presence of mold and dampness in the homes were correlated to a several respiratory and non-respiratory symptoms. The effect was of similar dimension to parental smoking (Brunekreef et al. 1989). Studies involving 15,000 children and 18,000 adults from 30 communities in Canada came to similar conclusions. A dose- effect was also seen in that more visible mold yielded more symptoms and a non-allergenic mechanism was involved (Dales et al. 1991a,b). Data from a further 13,000 children from 24 cites across the US and Canada show the same pattern (Spengler et al. 1994). The European studies are reviewed by Dales (1995) and Verhoeff (1994).

In these studies, self-reported respiratory symptoms were determined including cough, wheeze, asthma, bronchitis and colds. Similar health effects have been found both in children and adults and by different investigators using different questionnaires (Dales 1995). Those studies with objective measures of fungi gave positive associations between molds and health (Verhoeff & Burge 1997).

To pursue this, 400 homes were studied in Wallaceburg (Ontario). The Harvard-Health Canada mold/ dampness questionnaire was completed. Recordings of night-time cough were made and compared to data from the questionnaire. Environmental measurements were taken in the largest living area plus the child's bedroom. In both, 18 h air samples were analyzed for endotoxin and ergosterol. Swab samples were taken of visible mold. In the living area, a large dust sample was collected and analyzed for xerophilic and hydrophilic fungi, ergosterol, endotoxin, dust mite allergens and species, cat allergen and cell-line cytotoxicity. In the child's bedroom, dust mite allergens were determined in the mattress dust (Miller 1995). Approximately 270 species of molds were recovered from the dust although 20 were common. Excepting phylloplane species, this group included several moderately xerophilic and toxigenic *Penicillium* and *Eurotium* species. The xerophile *Wallemia sebi* was surprisingly common (Miller & Day 1997). The ratio of the sum of the colony forming units per gram dust *Aspergillus*, *Eurotium* and *Penicillium* divided by the sum of phylloplane species indicated that about 20% of the houses have unusually large exposure to the latter group. A similar proportion of houses had visible non-phylloplane mold growth from swab sample results (Miller 1995).

From the 400 homes, 39 were chosen with high objective measures of mold in the dust and air and 20 with correspondingly low values. Inspection revealed 0.42 m^2 and 1.2 m^2 visible mold in the low and high-mold homes, respectively. This was associated with a 25 fold increase in CFU/g dust on high water activity media, a 10 fold increase in the proportion of *Aspergillus* and *Penicillium* species to phylloplane species in the dust and detectable airborne ergosterol. The measurement of moisture source strength was highly correlated to mold CFU/g in dust. House age, heating system, general construction, occupant density, temperature and relative humidity were similar between the two groups. Air change rate was higher in the high mold homes. There were no significant differences in Der P plus Der F, cat antigen, dust and air endotoxin values, rates of combustion spillage, smoking prevalence and TVOC concentrations (Anon. 1995; Lawton et al. 1998). The visible mold data were incomplete because destructive testing was not done. In addition, tests of the distance that mold growth is present on building materials beyond the line visible to the naked eye under field conditions show that this is typically another 0.5 m (Miller et al. 1999).

Peak flows, respiratory symptoms, nasal lavage and extensive blood immunological measures were taken on the children. Gross symptoms followed the pattern of the epidemiological studies described above. Living in more, compared to less contaminated homes was associated with a larger number of CD3+ Tcells expressing CD45RO and CD20+Bcells expressing CD5+ and a reduced CD4/CD8 ratio. The levels of statistical significance were p=0.02, p=0.07 and p=0.06 respectively after controlling for child's age, dust mite antigens and the presence of furry or feathered pets or a humidifier. This represented two studies of the population taken within one year (Dales et al. 1998). This is the first biomedical evidence that the epidemiological indication of a linkage between residential mold exposure and health is real. This evidence suggests that the effect is associated with organisms that grow on building materials in the houses studied (as opposed to phylloplane species).

All these data provide convincing evidence that living in moldy homes produces health effects in children and adults. The data suggest that mold growth areas of 3-5% [of the type of molds that grow on building materials] of the total floor area resulted in the measured immune system changes. However, the attributable risk for mold growth for asthma, immune system or any other putative effect is not known. The maximum attributable risk for mold caused asthma from the Canadian 15,000 home studies was 20% (Dekker et al. 1991) but this estimate is based on questionnaire data.

USEFULNESS OF MOLD MEASURES

The American Industrial Hygiene Association "Field guide for the determination of biological contaminants in environmental samples" (Dillon et al. 1996) outlines a variety of methods for measuring fungi in air and on building materials. When these methods are used as described, reproducible data are generated. However, the scientific limitations of these methods were not reviewed. Indeed, there are no methods for measuring fungi in air or from building materials which are fully quantitative and qualitative.

Spores can be collected on sticky surfaces by impaction and then the surfaces are examined by microscopy, sometimes including one of a number of stains to help visualize different components of the flora. With short term samples, the quantitative data are of uncertain quality with larger, dark-colored spores being counted at the expense of small, hyaline particles. The qualitative measurements depend on the skill of the microscopist and are limited. Some samples are difficult to read because the microbes are obscured by inorganic particles.

Better qualitative information can be obtained by impaction on agar media or in liquid impingers. Such samples represent very short term collections (<5 minutes) and for several reasons including that there can be order of magnitude variation in spore concentrations in minutes, the numerical values are of limited use (Miller 1992). Different species of microorganisms have different growth requirements so the use of any medium produces different "recoveries". The spores of different species of microorganisms decline in viability with time; some spores remain viable for years and others for months. In general, the numbers of fungal propagules determined by culture are substantially less (1-50 %) than those determined by direct methods but this varies between species. Finally, some species are very "aggressive" in

culture and produce antifungal agents that affect the growth of others (e.g. *Trichoderma* species; see Taylor 1986). A longer review of these issues can be found in Miller (1992).

To illustrate this point, Table I illustrates the putative response of plating 1,000 spores of *Stachybotrys chartarum*, 40 spores of *Penicillium aurantiogriseum*, 20 spores of *Wallemia sebi* and 40 spores of *Trichoderma harzianum* on three media: malt extract agar (MEA), corn meal agar (CMA) and DG18 agar (Dillon et al. 1996). From the 1000 spores plated, 10 of *S. chartarum* appeared on CMA, the best medium of the three for this species or 1% of the total spores plated. Recoveries on the medium for moderate xerophiles, DG18 were very low. In contrast, a high percentage of the moderate xerophile, *P. aurantiogriseum* spores were culturable on its optimum medium, DG18. For the xerophile *W. sebi*, good recoveries were obtained on DG 18. Colonies of the hydrophilic, wood soft rot species *T. harzianum* is difficult to count under ideal circumstances and was poorly recovered on DG18.

TABLE I: Model data from plating four taxa on agar: Pure cultures.

	MEA	CMA	DG18	spores plated
S. chartarum	1	10	1	1,000
P. aurantiogriseum	10	20	25	40
W. sebi	1	1	10	20
T. harzianum	10	10	1	40

Table II illustrates the putative response of plating the mixture of species used to form the above example. *S. chartarum* recoveries even in the optimal medium used CMA are much reduced because of the presence of the presence of the *Trichoderma*. *W. sebi* is not reported at all and only the *Penicillium* species emerges "unscathed".

TABLE II: Model data from plating four taxa on agar: mixture of four species.

	MEA	CMA	DG18	spores in mixture plated
S. chartarum	5	5		1,000
P. aurantiogriseum	20	10	20	40
W. sebi				20
T. harzianum	10	10	1	40

These examples demonstrate the point that counting colony forming units on air or dust samples over-represent the long-lived tolerant *Penicillium* species. It also illustrates why normal descriptive statistics cannot be used on the numerical values present to any useful purpose: each sample is essentially unique and incomparable from a numerical perspective (Miller 1992).

A similar phenomenon occurs when dilution plating bulk samples such as soil or building materials such as wallboard except there is an additional difficulty. It has long been understood in soil mycology that dilution plating did NOT give a reliable indication of the species active in the ecosystem. Kjoller & Struwe (1984) review data from dilution plating soil samples over about one year period in comparison to measurements of hyphal growth in the soil. Typically, there were no correlations between the two such measures, the latter being a direct measure of

fungal activity. Fully 85% of the cultures found by dilution plating are of species not active in the ecosystem (Warcup 1965).

Colony Forming Unit data either from air samples or data from dilution plating of bulk sample have no inherent quantitative value. Dilution plating has considerable value in determining the total diversity present in a sample and there is some evidence that the data from many replicates produce data that are related to the absolute value (Brewer et al. 1980). The direct measurement and plating of bulk samples ("soil crumb method") is the most useful non biochemical method of measuring fungal activity in samples and this minimizes emphasis on species that are inactive in the system.

There is general agreement that properly conducted air sampling is useful for detecting fungal amplifiers but not for assessing exposure (Dillon et al. 1999). The numerical data obtained from culturable air samples has no value. A new study of these issues has been made by Miller et al. (1999) where the data from extensive air sampling in an apartment building was compared to studies of mold damage after massive destructive inspection to examine wall cavities.

As expected, the $CFUm^{-3}$ values between the two data sets were not significantly different using descriptive statistics. However, the proportion of samples that "fail" the above-noted AIHA guidelines was different ($p < 0.005$). When the data were compared to the area of visible mold revealed by the extensive destructive testing, there was no correlation with CFU m^{-3} values. The proportion of "AIHA fails" was fairly well correlated with measured mold area with p values of 0.03 to 0.10 depending on the stringency of the test (Miller et al. 1999).

Because of the need for true exposure measures, research on the effects of microbes in indoor air have mainly focussed on using indicator biochemicals that can be measured from particulate samples.

For fungi, many measurements in air have been made using (1→3)-β-D-glucan (Rylander 1998) and >1,500 have been made using ergosterol. Ergosterol is the primary membrane sterol of filamentous fungi and environmental yeasts and, with rare exceptions, is not accumulated by other organisms. This useful fact has encouraged a great deal of research using ergosterol as a biomass measure (Gessner & Newell 1997). A method was adapted for use in house dust (Miller et al. 1988) and data have been reported in other studies (Axelssen et al. 1995, Saraf et al. 1997). Ergosterol content of spores is comparable to values from mycelia and ergosterol contents are similar in different species that commonly grow on building materials. The analytical performance of the method has been characterized (Miller & Young 1997).

WALLACEBURG EXPERIENCE

We found that mold measurements in settled dust (CFU g^{-1}; 10 fold increase in proportion of moderate xerophiles) were correlated to moisture source strength (an estimation of water sources in a house) and related to mold area estimations by the inspectors (Lawton et al. 1998). Higher mold counts in settled dust were associated with occupant reports of moldy odors. Reported water damage was associated with increased mold counts in settled dust. *Aspergillus* and *Penicillium* species counts in settled dust were higher when occupants reported mold as not. Occupants who smoked or reported mold allergies under- and over-reported molds compared to objective data (Dales et al. 1997). Because of the problems of using dilution plating data as

exposure measures discussed above, these relationships are probably as good as they can be. Miller et al. (1988) and Saraf et al. (1997) showed that ergosterol (an absolute measure of fungal biomass in the sample) and CFU g^{-1} in settled dust were not well correlated. Douwes (1998) found that a similar measure to ergosterol, (1→3)-β-D-glucan and antibody-based methods for assessing *Aspergillus* and *Penicillium*, in settled dust were useful predictors of some health outcomes.

This means that at present, the best exposure indicators appear to be good estimates of mold area including a detailed assessment of the actual demarcation by culture studies (as opposed to that visible to the naked eye) and measurements of taxa present in settled dust plus ergosterol or glucan.

The measurement of airborne ergosterol or glucan appears to be a useful measure although information must also be collected on the taxa present by other means. Miller & Young (1997) demonstrated that the values of airborne ergosterol in the Wallaceburg houses were plausible but that the sensitivity of the assay was not sufficient to provide data from 18h samples at this time. Our future work in assessing fungal exposures for epidemiological studies has focused on good estimates of mold area, moisture source strength, ergosterol analyses of samples collected over a longer time period and mold in settled dust. These techniques together represent the best available tools but they are not sensitive enough for our purposes. More research is needed to produce exposure estimates of the quality ultimately obtained for aflatoxin after 30 years of work.

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FUNGAL GROWTH IN BUILDINGS: THE AEROBIOLOGICAL PERSPECTIVE

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ABSTRACT:

In the search for inexpensive shelter, we have developed indoor environments that are conducive to fungal contamination. While active fungal growth indoors is usually inappropriate and should be controlled, assessing specific health risks associated with such growth remains a challenge. Epidemiological tools are often used to determine relative risks associated with occupancy by groups of people in environments with or without certain factors, including fungal growth, but do not always make clear the role of the growth in the disease process in individuals. Aerobiologists assess relationships along a pathway that includes sources, dispersion and decay of aerosols, exposure to individuals, doses of agents, and responses. Both approaches yield valuable information, but require the development of testable hypotheses.

As a model, we can apply the epidemiological and aerobiological processes to the Cleveland hemosiderosis outbreak, and consider the following hypotheses:

1. The Cleveland babies that develop hemosiderosis are more likely to live in moldy homes than those that did not develop the disease, all other things being equal.

2. The Cleveland babies that developed hemosiderosis are more likely to live in homes with *Stachybotrys* than those that did not develop the disease, all other things being equal.

3. (Aerobiology) Babies that developed disease were likely to have received a dose of *Stachybotrys chartarum* toxins sufficient to cause the reported symptoms.

Some evidence exists to support Hypothesis 1. Hypothesis 2 is supported by very little evidence and Hypothesis 3 has not been tested. Until Hypotheses 2 and 3 are adequately tested and verified, assuming a cause/effect relationship for *Stachybotrys* toxins in these cases is premature. Premature establishment of cause/effect relationships may lead to unnecessary concern, and prevent discovery of actual causes of disease. This hypothesis development and testing process is essential if we are to accurately determine the role of indoor fungi in human disease.

THE SCIENCE OF AEROBIOLOGY

Definition

Aerobiology can be defined as the study of airborne particles of biological origin and their effects on living organisms. The science uses information and practices from many diverse disciplines, including microbiology, mycology, aerosol physics, meteorology, toxicology, medicine,

epidemiology, statistics, and probably others. Practitioners of any of these disciplines might do aerobiological research, or use the principles of aerobiology in the practice of his/her profession. A person whose principal activities involve aerobiological research must be trained to some extent in all of these disciplines.

Relation of Aerobiology to Other Health-Related Disciplines

Most aerobiology disciplines use hypothesis generation and/or testing to gain information about the relationship between exposures and disease. Hypothesis generation involves asking questions about a particular outcome, developing possible answers to the questions (the hypotheses), and then challenging the null hypotheses. In other words, one tries to prove that the answers are wrong. If such proof is not possible, then the hypothesis is considered to be true. Although this seems like a backwards approach, it is, in fact, extremely important, since it lessens the chances that the testing (the study design) will be biased in favor of proving the hypothesis true. Although the process of hypothesis generation and testing is common to most disciplines, the types of hypotheses generated and the way they are interpreted may differ. For example,

Environmental epidemiologists develop hypotheses about the relationships between environmental factors and the presence of disease in large populations, using rigorous standards to test the validity of these hypotheses.

Laboratory Scientists develop hypotheses about causes of disease and test these hypotheses in laboratory animals, isolated cell systems; etc., also using rigorous standards to test the validity of the hypotheses.

Clinical practitioners care for individual patients, attempting to determine the most likely cause of disease in the patient. Clinicians do not test hypotheses, although they often must assume that even untested or unproven hypotheses are true.

Building science practitioners investigate buildings and do not generally test hypotheses. However, they use existing hypotheses to guide decisions for remediation; like the clinician, they sometimes must assume that untested or unproven hypotheses are true.

Aerobiologists: Develop hypotheses about the causes of airborne disease (based on evidence from clinical medicine and epidemiology). They use laboratory science (microbiology, physics, human physiology, toxicology) to test these hypotheses, and, in collaboration with risk assessors, develop exposure guidelines.

The Aerobiological Pathway

Aerobiology is primarily concerned with disease that is contracted via inhalation of agents transported through the air, and it is useful to consider the pathway by which such diseases are produced.(Figure I). By tracing supporting evidence through this pathway,one can make conclusions about the reliability of particular hypotheses with respect to such diseases.

FIGURE I:

Source Factors	➢ Aerosol Factors	➢ Exposure Factors	➢ Dose/Response Factors
Organisms and agents *physiology* *biochemistry*	Aerosol release Composition Dispersion Physical decay	Time Activity Inhalation factors	Cellular responses Systemic responses
Populations *interactions* *dynamics*	Biological decay	Deposition, removal factors Agent release and transport	Other factors Immunology Toxicology

Pathway complete (Type 1 condition): The aerobiological pathway is complete if all factors support a cause/effect relationship between the source and the response. In this case, the designated agent or source is *likely* to be at least one of the contributing factors to disease, although other factors may play a crucial role. Additional research would appropriately focus on identifying these other factors.

Pathway incomplete (incomplete data) (Type 2 condition): An incomplete pathway can result from incomplete data or from data that does not support one or more steps in the pathway. In the case where data are incomplete, the agent *may* be at least one of the contributing factors to disease, and additional data must be collected to document the relationship. This is one of the situations where a clinician or a building scientist might consider the pathway complete (the untested or unproven hypothesis true) until confirmatory or contrary data become available. In these cases, one must always remember that the hypothesis may not be true, and that other factors may play a crucial role in the disease process. This type of pathway break clearly identifies areas for further research.

Pathway incomplete (data indicate break in the pathway) (Type 3 condition): If data are generated that clearly break the aerobiological pathway, the agent must be considered *unlikely* to play a role in the response (e.g., the disease outbreak), and other factors should be sought. In this case, it is not appropriate to assume that the hypothesis is true.

EXAMPLE: THE ROLE OF S. CHARTARUM AS AN INHALANT TOXIN IN BUILDINGS

SOURCE FACTORS

Organisms and agents

Many different isolates of *S. chartarum* have been studied for toxin content, and an array of toxins has been identified (Bata et al. 1988; Jarvis 1995). Most are macrocyclic trichothecenes. Most strains accurately identified as *S. chartarum* produce some toxins at least in the laboratory (Nikulin et al. 1994). However, the amount of overall toxicity and the array of toxins produced are strain-dependent. There is also some evidence that toxin production may be poor on some substrates. However, overall, when *S. chartarum* is found growing on a substrate, the chances

are reasonably good that toxin is being produced. This is supporting evidence in the aerobiological pathway linking *S. chartarum* growth in buildings and an ultimate response following exposure.

Populations

Population dynamics for *S. chartarum* have not been reported. Evidence has been presented that *S. chartarum* requires wet conditions (Grant et al. 1989; Flannigan et al. 1991). It also has been shown to use cellulose as a carbon source, and to compete well when few other nutrients are available. Tested strains also do well under cool conditions (<20oC). Growth on indoor building materials, particularly wallboard, produces abundant conidiophores and spores. Thus, if conditions continue to be wet and cool, *S. chartarum* growth may continue until nutrients in the wet material become limiting. However, without repeated direct measurement of either these conditions or the fungus over time, the strength of the reservoirs will be unknown. In addition, the kinds of organisms sharing habitats with *S. chartarum* remain unknown, as are the effects of competition with these organisms on growth, sporulation and toxin production by *S. chartarum* (Butt & Ghaffar 1972). These factors may indicate a Type 2 break in the aerobiological pathway, and additional data may be needed to connect a source measurement with a response.

Aerosol factors

Aerosol release

S. chartarum spores are hydrophilic, and tend to stick together. Studies of the dynamics of spore release into the air have not been reported. Ongoing studies in our laboratory indicate that strong mechanical disturbance is necessary for spore release, and that even with such disturbance only a very small fraction of available spores actually enters the air. This is evidence that indicates a possible Type 3 break in the aerobiological pathway, and serious consideration needs to be given to the possibility that *S. chartarum* is not the culprit.

Composition/nature

The only aerosol particles identified as *S. chartarum* are spores, which range from 3-4 x 7-10 μm with extensive variation among different isolates (Korpinen and Uoti, 1974). Other toxin-bearing particles could be released from surfaces, but have yet to be identified. Aerosol concentration dynamics depend on the sizes of the aerosol particles. Until the toxin-containing particles have been clearly defined, aerosol concentration dynamics cannot be predicted. Also, the stability of toxins either within substrates or in air has not been reported. These factors represent Type 2 breaks in the aerobiological pathway, and indicate fruitful areas for research.

Exposure factors

Time, activity

Time spent in a particular environment and activity while present in the environment (both of which affect the number of particles that can be inhaled) can be documented with reasonable accuracy, although such documentation has not been a part of reported cases of *S. chartarum* poisoning. This represents a Type 2 break in the pathway for the published literature, although on that could be readily eliminated for future studies.

Inhalation, deposition factors

The physics of particle inhalation and deposition in human lungs is well characterized (Leong et al. 1998). If *S. chartarum* toxins are primarily associated with spores, then relatively few particles penetrate beyond the upper airways (Sorenson et al. 1987). If the effects reported depend on lower airway deposition, then this represents a Type 3 break, and alternative causes for disease processes should probably be sought unless doses required are very low (see below). Effects of upper airway deposition have not been studied. If one hypothesizes that the upper airway is an effective route of exposure, then this break would revert to Type 2, and additional data on such effects should be sought. However, until the actual characteristics of toxin aerosols are clearly documented, these factors cannot be predicted, and, clearly, a Type 2 break is present.

Removal and agent-release factors

Removal rates from the lung for inert particles are well known. However, the effect of toxins on removal rates is unknown for *S. chartarum* spores or for most other toxin-containing particles. In addition, for particulate toxins, both the amount of toxin released and the rate of release of the toxin from the particle is an important parameter that remains unknown for *S. chartarum* spores or other toxin-containing particles. These represent Type 2 breaks and indicate useful areas for research.

Response factors

Responses

Cellular responses are well documented in laboratory animal and in-vitro studies. *S. chartarum* produces potent cytotoxins that lead to release of inflammatory mediators and to cell death and hemorrhage, at least when large doses are administered (e.g., Nikulin et al. 1997; Sorenson et al. 1986). Systemic effects from these large doses have also been reported. However, although ingestion of food colonized with *S. chartarum* clearly produces symptoms in people, inhalation exposures that can be unequivocally said to result in adverse health effects have not been reported. One study provides reasonable evidence for such effects (Croft et al. 1986) although alternative causes for symptoms (e.g., exposure to other agents, non-environmental causes) were not extensively explored. In addition, further confirmatory documentation of similar effects in other *S. chartarum*-contaminated environments has not been forthcoming. This may be interpreted as a Type 2 break if inadequate study design has prevented such documentation.

Dose factors

It is clear that in laboratory animals, acute one-time exposure to *S. chartarum* spores must be very high ($\sim 10^6$ spores) to elicit release of inflammatory mediators and do sufficient damage to the epithelium to cause hemorrhage. Lower doses have produced little or no effect in exposed animals, at least with respect to the measured parameters (e.g., Nikulin et al. 1997). This, combined with the low aerosolization potential for *S. chartarum* spores, indicates a clear Type 3 break for responses to acute challenges such (for example) as might occur when an investigator enters an *S. chartarum*-contaminated building. However, low level chronic exposure effects have not been studied. This is a Type 2 break in the pathway, and a crucial area for additional research. In addition, all data on *S. chartarum* effects has been derived from rodent mod-

els, and approaches for extrapolation from these models for this particular toxin have not been formulated. This is another area that urgently needs research.

CONCLUSIONS

Although several reports cite *S. chartarum* as the agent for building-related symptoms, none provide evidence that completes the aerobiological pathway from source to exposure (Cooley et al. 1998; Montana et al. 1997). Thus, for *S. chartarum* as a cause of building-related illness, the aerobiological pathway is far from complete. There is reasonable evidence that aerosol release from *S. chartarum* colonies is minimal, and that, except under very active disturbance, aerosol levels are unlikely to reach concentrations that would lead to significant pulmonary deposition in either adults or children. For hypotheses not depending on pulmonary deposition, many Type 2 breaks exist in the pathway, and much research remains to be done before one can assert that *S. chartarum* is an important, or even a likely cause of building related illness.

The aerobiological pathway provides a useful stepwise approach for designing studies and for interpreting the literature on the causes of airborne disease.

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WHY ARE THERE STILL PROBLEMS WITH FUNGAL ALLERGEN EXTRACTS?

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INTRODUCTION

It has long been axiomatic that reliable allergen extracts of fungi are more difficult to produce and work with than extracts of pollen, dander, or other allergen sources (Salvaggio, 1981; Burge, 1985; D'Amato, Spieksma, 1995; Feinberg, 1946). Features of fungi that contribute to this include their number and variety, the identification is sometimes difficult, their phenotypic plasticity, and the presence of endogenous proteases that can degrade allergens in crude extracts. All of these problems can be addressed to an extent, albeit some with difficulty. Indeed, with the few fungi with which molecular techniques are being used, recombinant allergens are readily being produced. Unfortunately, adequate resources and resolve are lacking for a concerted effort at producing well-characterized allergen extracts. Further, the extracts that are available correspond poorly with the fungi that are often a problem indoors.

WHY DO WE NEED RELIABLE FUNGAL EXTRACTS?

Allergen extracts are solutions that contain the IgE reactive antigens from an allergenic material. Extracts of many allergen sources - but no fungi - are now available in standardized form with clearly defined amounts of the major allergens. Allergen extracts are needed in skin prick tests (SPT), in radioallergosorbent tests (RAST), and IgE ELISAs to determine if subjects are sensitized to an allergen. These tests detect allergen-specific IgE which indicates sensitization. Knowledge of sensitization greatly aids allergy diagnosis.

Several issues are of concern though, for diagnostic testing with allergen extracts. False positive and false negative reactions are a concern with allergen testing as with all diagnostic procedures. If the potency of an extract is insufficient to elicit reactions in sensitized subjects, then sensitized subjects may not be detected. The allergen concentration - and hence potency - of many fungal extracts is often low. This has long been a recognized problem with mold allergens, and is likely to only be addressed as recombinant materials become available. Conversely, extracts with irritants may induce false positive reactions. Extracts should always be tested in non-atopic subjects to detect any non-specific reactions, and so avoid false positive reactions. In skin testing, intradermal (ID) tests are more sensitive than skin prick tests (SPT); however, SPT tests are often preferred for diagnosis since ID tests are also more prone to false positive reactions.

Comparable problems can be encountered in RAST or other in vitro tests. False positive reactions are avoided by testing extracts against panels of sera from subjects that are allergic, but not to molds. Reactions must exceed this range by some amount (e.g. 2 standard deviations) to be considered positive. It should also be noted that SPT is generally more sensitive than RAST or IgE ELISA, so negative in vitro results should be confirmed by SPT where indicated.

In addition to B cell epitopes that bind IgE, allergens also contain T cell epitopes which allergen-specific T cell responses are directed against. It is now recognized that the antigen -specific Th1 / Th2 polarization is central to whether allergic reactions occur (Huang1998). Hence, characterizing T cell epitopes is key to understanding the nature of allergies. Major allergens are best selected from well-characterized extracts for T cell epitope studies.

Allergen extracts are also needed for immunotherapy, where deemed appropriate. Traditional immunotherapy relies solely on allergen extracts, usually crude extracts for fungi. Recent advances in understanding the importance of T cell epitopes underlie new immunotherapy strategies based on modulating the T cell responses to an antigen without triggering B cell responses (Platts-Mills et al. 1998). These strategies also require identification and characterization of the major allergens and their epitopes, though, which requires potent, well-characterized extracts.

The production and especially screening of allergen-specific monoclonal antibodies (mAbs) also need extracts. Such mAbs are useful as the basis of immunoassays for the environmental assessment of indoor and outdoor allergens, including fungi. In fact, environmental immunoassays for fungal allergens would be very useful as exposure indices.

WHY ARE FUNGAL EXTRACTS SO DIFFICULT TO CHARACTERIZE AND STANDARDIZE?

Indoor fungal exposure is more complex to assess than for other allergens. There are two or three important mite species, two important cockroach species and one species each of cat and dog. Only exceptional buildings would have significant concentrations of all six or seven of these "indoor allergens." However, the indoor environment of a damp building commonly may have three or four *Penicillium* species, perhaps as many *Aspergillus* species and these mixed among another five to ten species of other fungal genera (Hoekstra et al. 1994). Thus, the potential fungal exposure is far more varied than to other allergens.

The allergen composition of individual species is also more complex than other allergen sources, indoors or out. There is one major cat allergen, two dust mite allergens account for most mite reactivity, and there are about five major cockroach allergens. Fungal species, however, typically contain numerous allergens. *Pleurotus ostreatus* and *Psilocybe cubensis* spore extracts were each reported to have at least 13 allergens (Horner et al. 1988; Helbling et al. 1993). Six allergens have been sequenced (at least partially) from *Alternaria alternata* and at least 10 each from *Cladosporium herbarum* and *Aspergillus fumigatus* (Achatz et al. 1995; Crameri,1998).

The variability of fungal allergen extracts is another problem in characterizing fungal allergens. Several factors contribute to this variability. One is the difficulty of collecting pure spores of many fungi, particularly ascomycetes and some basidiomycetes. Without adequate spore quantities, extracts are made from mycelia. Although mycelia are easy to grow for many species, the

antigen content of mycelia usually differs from that of spores (Helbling et al. 1993; Hoffman et al. 1981). This is significant since most fungal exposure is to spores. Further, different strains of a single species can vary and these differences must be considered. Fungi also vary dramatically according to their growth stage the substrate on which they are cultured, and the growth conditions. Thus, scrupulous detail is required to produce consistent batches of extracts because source material, strain selection, and culture conditions all impact the variability of fungal extracts (Helm et al. 1987).

Although relatively few fungal extracts have been tested for protease activity, those that were tested have demonstrable activity (Esch, 1992; Wongtim et al. 1993). The proteases themselves are likely allergens in some cases. More importantly, the presence of proteases has implications for the stability of fungal allergen extracts because the proteases may degrade other allergens in the extract. In fact, until recently, mold, pollen and other allergen extracts were commonly mixed for immunotherapy. Mold (and cockroach) allergens are now usually excluded from any extract mixtures to avoid compromising the potency of the extracts.

Fungal identification can be difficult without specialized training. This is sometimes not recognized, and incorrect identifications are made. Other times, expertise is unavailable and fungi are identified only to the genus rather than the species level, or they are simply counted with no identification attempted. These difficulties have long prevented rigorous analysis of environmental fungi, especially indoors.

The indoor mycota is now well described (Hoekstra et al. 1994), but in many early (and some current) allergy studies, the fungi were incorrectly identified and these studies sometimes focused on minor rather than dominant components of the indoor environment. Unfortunately, the selection of indoor fungi for producing allergen extracts was often based on these early studies or on outdoor fungal studies. The fungi that typically occur indoors should now be used to better reflect indoor exposure. Extracts from some of these species are not commercially available and better quality extracts are needed overall for fungi. However, developing new extracts is not cost effective since there is a high regulatory burden and fungal extracts are only a small segment of the allergen extract market, which is itself a small market within the pharmaceutical industry.

HOW ARE ALLERGENS CHARACTERIZED?

Allergens are IgE-reactive antigens and, hence, are characterized by their reactivity with human IgE. They are typically extracted in an aqueous buffer solution from a suitable source material. RAST, IgE ELISA, and immunoblotting are widely used to study allergen extracts; therefore, central to any characterization is a pool or panel of sera from subjects who are sensitized to the allergen of interest. Traditionally, crude extracts were prepared and used to identify highly sensitized subjects. Sera from these subjects were used to prepare partially purified extracts, which were then used to obtain a wider variety of allergic sera. Obviously, a larger panel of sera gives a more thorough detection of allergens and more definitive identification of the major allergens.

The allergens that react with a majority (>50%) of sensitized subjects' sera are considered major allergens. This is a relatively straightforward concept with many allergens. Identifying

major fungal allergens is often a substantial challenge though, since there are generally more allergens in an extract, and broader diversity of reactivity among sera. When the major allergens have been identified, mAbs typically are prepared which can relieve the dependence on sera from allergic subjects. It remains essential though to verify that the mAbs recognize the human allergen, because mice do not always react to the same components as humans. Also, because these antibodies may react with epitopes other than the IgE-binding epitopes, such reactivity may not reflect the activity of an allergen.

Molecular genetic techniques now used to produce expression libraries and phage display systems efficiently screen allergen sources for allergenic proteins (Baldo, Donovan, 1988; Scheiner,Kraft, 1995). Further, when allergen producing clones are recovered, purified allergen can be isolated from the clones far more easily than from crude source materials. Although these superior techniques are now commonplace for the study of many aeroallergens, their application to fungal allergen analysis is still scarce. Sequence data have been reported from 17 fungi, but 8 of these are very rare indoors and another 2 usually occur outdoors. Obviously, this is a barely tapped research area.

WHAT IS AN APPROPRIATE STARTING MATERIAL?

Spore inhalation is probably the major means of exposure to fungal allergens. Hence, the allergens in spores are presumably the allergens of greatest clinical relevance. Although not abundant, hyphal fragments do routinely become airborne and fragments of hyphae and/or particles of substrate colonized by fungi that are incorporated into dust may also be sources of fungal allergens.

Culture fluid or "spent' culture broth has often been used as a crude source material for fungal allergens. Its advantage is that large quantities can be very easily produced. However, there is a major concern when using culture fluid. Since fungal metabolism is dramatically influenced by growth conditions, the enzymes being expressed and the proteins being secreted may vary dramatically between pure cultures during exponential growth and cultures in nature growing under limiting conditions and competing with other organisms. Since allergens have been recovered from fungal culture fluids, clearly some fungi release allergens under these conditions (Lopez et al. 1976). In fact, one *Aspergillus fumigatus* allergen that is known to be involved in allergic bronchopulmonary aspergillosis is secreted into culture filtrate (Bannerjee et al. 1977). Most allergen extracts use mycelia or spores as source material, though.

A significant proportion of mold allergen research has been conducted on mycelial extracts. Indeed, some allergens do not occur in spores and are produced and released only after germination (Arruda et al. 1992; Sporik et al. 1993). However, there are also spore-specific allergens (Helblinget al.1993: Hoffman et al. 1981; Aukrust et al. 1985; Weissman et al. 1987), and, since the principal exposure to fungal material is likely from spores, they are the preferred source material for fungal allergen extracts.

The allergens in an extract should reflect the complement produced in the environment. Fungi are phenotypically plastic, so fungal metabolism can alter significantly depending upon growth conditions. The extent of this change in allergen profile and the conditions needed to control

this must be rigorously established. In general, culture conditions should reflect the conditions typically encountered in the environment.

PANELS OF WELL-CHARACTERIZED SERA

Well-characterized sera from sensitized subjects are required for allergen analysis. Although a panel of mono-sensitized sera would be ideal, subjects who are only sensitized to fungi are relatively scarce. Subjects allergic to fungi are detected with the available commercial extracts; these usually have low sensitivity, and many sensitized subjects are likely missed. Regardless of the complications, allergen identification, epitope mapping, and even, as discussed above, verifying mAb reactivity require a panel of sera.

CROSS-REACTIVITY

Cross-reactivity results when common epitopes occur among allergens in different extracts. Among plant allergens, this is well documented for sources as disparate as latex and banana. The occurrence of antigenic similarity that is sufficient to permit cross-reactivity suggests that these are highly conserved proteins. Indeed, recent evidence indicates that these allergens are plant defense enzymes; these would be expected to be highly conserved (Blanco et al. 1999). Among yeast, the major allergen is enolase, also a conserved enzyme (Ito et al. 1995). Baldo even suggested that commercial preparations of fungal enzymes might be useful source materials for fungal allergens (Baldo, 1995). This proposal likely has merit since IgE reactivity to these enzymes is common among mold allergic subjects (Horner et al.1997) and DNA sequences are known for many of these enzymes.

Clinically, cross-reactivity of allergen extracts is very important. Cross-reactivity may cause SPT or RAST reactions to allergens that the subject is not exposed to. This could lead to a misdiagnosis if IgE reactivity was relied on exclusively or if the clinical history were inconclusive or not carefully taken. Conversely, if cross-reactivity patterns of fungal allergens are sufficiently broad, then a panel of well-characterized extracts of fewer species may adequately diagnose fungal allergies within a group of species. This would also mean, though, that regardless of which species a subject was originally sensitized against, subsequent exposure to any of the group would possibly initiate an allergic reaction.

The patterns of cross-reactivity are important in pollen allergy and food allergy, among others. Presumably, these cross-reactions are also significant in fungal allergy. The limits and extent of these patterns must be determined as they have been for grass pollens, if fungal allergy is to be properly managed. Several studies have shown substantial cross-reactivity among members of related species and even genera (DeZubiria et al. 1990). One study compared the major groups of fungi, but only minimal cross-reactivity was shown (O'Neil et al.1990). Since the cross-reactivity patterns for fungi are incompletely known, the elucidation of these patterns should be a high priority.

CONCLUSION

Numerous persistent problems hinder the production of well-characterized, potent fungal allergen extracts. Some are inherent to the fungi, others are basically due to researchers= choices. Although fungal variability cannot be changed, applications of mAbs and molecular techniques promise to reduce the problems due to fungal variability. Fortunately, strategies and techniques that have been useful with other allergens give ample precedence for their use with fungi.

Those problems due to researchers, such as choice of species, can and should be corrected now. The choice of species is principally a problem with exposure to the fungi that amplify indoors, although basidiospores are a major outdoor fungal exposure source and extracts for these are essentially non-existent. The indoor fungi are now well known and test panels should be developed with extracts of these fungi since many are not available commercially. Such extracts should be well characterized and the patterns of cross-reactivity elucidated. This will permit researchers and clinicians to better assess the prevalence of fungal allergy and help distinguish between allergic and non-allergic mechanisms related to the respiratory symptoms associated with damp buildings.

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FROM FUNGAL EXPOSURE TO DISEASE: A BIOLOGICAL MONITORING CONUNDRUM

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ABSTRACT:

Biological monitoring is the estimation of exposure to an agent through the measurement of biomarker(s) resulting from an internal dose of an agent. The biomarker(s) are typically the agent or its metabolite(s) in a biologically derived specimen. For example, styrene in expired air, styrene in blood, and mandelic and phenylglyoxylic acids (metabolites of styrene) in urine. The biomarker also can be a highly specific, validated effect of the agent, such as elevated levels of zinc protoporphyrin in blood caused by exposure to lead. Due to advances in molecular biology, genetics, analytical chemistry, and other basic sciences, it is now possible to detect smaller amounts of analytes and contaminants and smaller biological changes, as well as to identify mechanisms at the cellular and molecular levels. These advances can be exploited for biological monitoring, in some cases. Legacy biological monitoring, is the measurement of a specific biological monitoring determinant which contains none of the atoms of the molecule to which the organism was exposed. The most common example of this is an antibody produced against small molecular weight molecules or altered constitutive proteins. Numerous investigators have implicated putative exposure to macrocyclic tricothecenes and other compounds from *Stachybotrys chartarum* as being associated with a plethora of signs and symptoms of disease. To date, no compelling biological marker for exposure to *Stachybotrys chartarum* mycotoxins or other metabolites has been presented. There is no doubt that exposure to *Stachybotrys chartarum* is associated with an increased prevalence of self-reported symptoms. However, evidence of the association of these complaints with frank disease is lacking. More biological monitoring research is needed to solidify these associations, if present.

KEY WORDS:

Biological Monitoring; Legacy Biomonitoring; *Stachybotrys chartarum*

INTRODUCTION

In general, an exposure to a chemical doesn't necessarily mean the existence of a body burden of the agent. In order for a chemical to gain entrance into the body it must be absorbed. Depending on the physical and chemical characteristics of the substance, absorption can occur through dermal, inhalation, ingestion or a combination of routes. Whether or not absorption from a chemical exposure occurs depends on the chemical properties, in general, related to its

lipid solubility. Once absorbed, a chemical is distributed and partitions into various tissues due to tissue variations in pH, permeability, etc. More water soluble chemicals are absorbed throughout the total body water, while more lipophillic substances tend to be distributed in body fat. The loss of chemical from the body can loosely be defined as elimination, which depends on metabolism and excretion. Chemicals may be eliminated by numerous routes including fecal, urinary, exhalation, perspiration and lactation. A chemical can be excreted from the body without metabolism, in which case the parent chemical will be detectable if analytical methods exist. In other cases, the chemical may be metabolized, which is the process of alteration of the chemical in the body. Metabolism may occur in numerous body tissues including liver, kidney, brain, etc. In most cases, metabolism is the result of oxidation, reduction, hydrolysis, or combination of these processes followed by conjugation; however, direct conjugation with an endogenous substrate is also a pathway for excretion. Most important conjugation reactions include glucuronidation, amino acid conjugation, acetylation, sulfate conjugation or methylation. Glucuronidation is the most common metabolic pathway. Metabolism/excretion and the rate of metabolism/excretion can be affected by age, diet, general health status, race, as well as other factors. In general, the metabolized chemical will be more water soluble than the parent. Also, there may be more than one type of metabolite produced from exposure to one parent (i.e., parent-glucuronide, parent-sulfate, etc.). The amount and ratios of parent-metabolites produced are affected by an individual's general health status, diet, nutrition, degree of hydration, time after chemical exposure, etc. In general, the kidney is the major organ of excretion and is the primary route of excretion of low molecular weight water soluble substances. These substances are excreted into the urine by glomerular filtration and tubular reabsorption as well as tubular secretion (Droz, 1989). Chemicals may also be excreted in the bile, which will not be covered in detail here.

DISCUSSION

Blood represents the transport vehicle for chemicals and their metabolites in the body. Therefore, most determinants present in the body can be found in the blood. Measurements taken from peripheral blood represent the levels of determinants during the short period of sampling (Fiserova Bergerova et al. 1989). The advantages of blood monitoring are the relatively small inter-individual variation of those blood components which affect blood levels of most determinants, and a simple sampling technique which provides relatively little opportunity for contamination. The disadvantages are: 1.) The sampling technique is invasive and can be performed only by medical personnel. 2.) The samples deteriorate when not properly stored and transported. 3.) Analytical procedures usually require meticulous cleanup procedure(s). The determinant's level in blood tends to equilibrate with its level at the site of its entry in the body as well as with its levels in tissues and excreta. Therefore, depending on existing body burdens, the blood level of the determinant may vary from one blood vessel to another. The level at the site of entry can be given as the concentration in ambient air (in case of inhalation exposure), in a vehicle (in case of dermal exposure), or metabolite's concentration at metabolic sites (usually in the liver) (Fiserova Bergerova et al. 1989). For the purpose of biological monitoring, the concentration differences in blood collected from different blood vessels can be significant on two occasions: 1.) blood collected from an arm which has been in direct contact with

a chemical may have a higher concentration of the chemical than blood collected from another site (Fiserova Bergerova et al. 1989). Blood flow carries the chemicals or their metabolites from sites of entry (usually lung or skin for parental chemicals and liver for metabolites) to other parts of the body. The transportation rate depends directly on perfusion of the organ. The rate of transfer of the chemicals or their metabolites from blood to tissues (i.e., tissue uptake rate) depends on the ratio of their solubility in tissue to that in blood (Lowry et al. 1989). For example, high lipid solubility prompts the accumulation of organic solvents in body fat. The binding of the chemical or metabolite to a specific tissue constituent results in its accumulation at the binding site (e.g., binding causes accumulation of cadmium in kidneys and fluoride in bone). Uptake of a chemical by the excretory organs is relatively high due to a large concentration gradient caused by removal of the chemical by excretion and metabolism. After the exposure ceases, the determinant level in blood declines due to elimination. When the blood level drops below tissue levels, the transfer process reverses and the determinant is washed from the tissue into the blood. Thus, determinants carried by blood from tissues with a higher saturation level are partly taken up by tissues with a lower saturation level (which is the redistribution process) and partly removed from the body by excretion or metabolism (which is the elimination process) (Lowry et al. 1989). Since levels of free (unbound) determinants in tissues and blood readily equilibrate, measurements of determinants in venous blood reflect the level of free determinant in the tissue perfused by the blood specimen. Since levels of the determinant in all tissues are not necessarily the same, the measurements in blood depend on the collection site and on the level of perfusion of the site. The mean level can be measured in arterial blood or in mixed venous blood. However, sampling of such blood specimens is not practicable in the field (Lowry et al. 1989).

Urine is more suitable for monitoring hydrophilic chemicals, metals and metabolites than for monitoring chemicals poorly soluble in water. The concentration of the determinant in urine usually reflects its mean plasma level during the period the urine dwells in the bladder (Rosenberg et al. 1989). In some instances, however, the urine concentration is also affected by the amount of a determinant stored in the kidneys. The advantages of urine monitoring are noninvasive sampling technique, availability of sufficient volume of the sample, and requirement of relatively simple clean up procedures. The main disadvantage is the variability of urine output which is determined by water intake and loss, which is influenced in turn by the ambient temperature and humidity. Contamination during sampling can occur and can be critical for measurements of small levels of parent chemicals. Moreover, urine levels of determinants are significantly affected by the industrial environment. The accuracy of the exposure estimate using urine monitoring depends upon the sampling strategy. The most influential factors are time of collection and the urine output. To reduce the effect of urine output, the measurement of the excretion rate instead of concentration is advocated (Rosenberg et al. 1989). Spot samples, representing urine output for two to four hours, are conveniently obtained and stored, and they provide a sufficiently large volume for analysis. The measurements in these specimens, collected at the identified time with respect to the exposure, usually provide good information on the intensity of the exposure. Determinant levels, however, can be affected by the length of sampling if the excretion rate changes rapidly during the sampling period (Rosenberg et al. 1989). Measurements in 24hour specimens are more representative than spot samples and usu-

ally correlate better with intensity of exposure. However, collection and transportation of these specimens are troublesome and rarely possible in the field (Rosenberg et al. 1989).

Urine measurements can be expressed as concentration (mg/L), or they can be related to urine components, whose excretion rates fluctuate less than water excretion. Such components are total solids (measured by specific gravity) or creatinine. Measurements in specimens collected for a known period of time can also he expressed as excretion rate (mg/hr) (Rosenberg et al. 1989).

BIOLOGICAL MONITORING OF EXPOSURE

Rational biological monitoring is only possible when sufficient toxicological information has been gathered on the mechanism of action and/or the metabolism (absorption, biotransformation, distribution, excretion) of xenobiotics to which individuals may be exposed. When a biological monitoring method is based on the determination of the chemical or its metabolite in biological media, it is essential to know how the substance is absorbed via the lung, the gastrointestinal tract, and the skin, and subsequently how it is distributed to the different compartments of the body, biotransformed, and finally eliminated. It is also important to know whether or not the chemical may accumulate in the body. These different kinetic aspects must be kept in mind when selecting the time of sampling. Biological monitoring of exposure is of practical value only when certain relationships between external exposure, internal dose, and adverse effects are known. Normally, biological monitoring of exposure cannot be applied for assessing exposure to substances that exhibit their toxic effects at the sites of first contact (e.g., primary lung irritants) and are poorly absorbed. In this situation, the only useful quantitative relationship is that between external exposure and the intensity of the local effects (Lauwerys, Hoet, 1993). Usually, for such substances the internal dose cannot be quantitatively related to the risk of adverse effect. For the other chemicals that are significantly absorbed and exert a systemic toxic action, a biological monitoring test may provide different information. If only the relationship between external exposure and the internal dose is known, the biological parameter can be used as an index of exposure, but it provides little information on the health risk. If a quantitative relationship has been established between internal dose and adverse effects, i.e., if the internal dose effects and the internal doseresponse relationships are known, biological monitoring allows for a direct health risk assessment and thus for an effective prevention of the adverse effects. Exposure to chemicals which modify the activity of the biotransformation enzymes (e.g., microsomal enzyme inducers or inhibitors) may also influence the fate of another compound (Lauwerys, Hoet, 1993). Furthermore, metabolic interferences may occur between chemical exposures and alcohol, food additives, pesticide residues, drugs, and tobacco. Several biological conditions (sex, weight, fatty mass, pregnancy, diseases, etc.) may also modify the metabolism of an chemical (Lauwerys, Hoet, 1993). In summary, the relationship between total uptake of an chemical, its concentration or that of its metabolites in biological media, and the risk of adverse effects may be modified by various environmental and biological factors. They may have to be taken into consideration when interpreting the results of biological exposure tests. Whatever the biologic parameter measured (the substance itself, its metabolite(s), or a biological effect), the test must be sufficiently sensitive (not too many

false negatives) and specific to be of practical value (not too many false positives) (Lauwerys, Hoet, 1993).

BIOLOGICAL MONITORING OF EFFECT

Health surveillance must be clearly distinguished from ambient monitoring and biological monitoring of exposure. Health surveillance evaluates the health status and aims at identifying individuals with early signs of adverse health effects, i.e., effects which are likely to be reversible or which do not progress to significant functional impairments when the exposure conditions are improved (Lauwerys, Hoet, 1993). Health surveillance must also be distinguished from the diagnosis of exposure- related diseases, which are the consequence of inadequate prevention programs. There are three types of monitoring (ambient monitoring, biological monitoring of exposure, and health surveillance); and these three types of monitoring are often applied simultaneously. Many chemicals may exert both local and systemic effects. The prevention of acute toxic effects on the respiratory tract or on the eye mucosa can only be prevented by keeping the airborne concentration of the irritant substance below a certain level (Lauwerys, Hoet, 1993). Likewise, biological monitoring is not usually indicated for detecting peak exposure to dangerous chemicals. On the other hand, an internal dose of a chemical considered safe by reference to the currently proposed biological limit values may still cause harmful effects in particularly susceptible individuals (Lauwerys, Hoet, 1993). Furthermore, the distinction between adverse and non-adverse biological effects is not always clear cut. Biological monitoring of exposure attempts to estimate the internal dose. But depending on the chemical and the analyzed biological parameter, the term internal dose may cover different concepts. Internal dose may mean the amount of chemical recently absorbed. Hence, a biological parameter may reflect the amount of chemical absorbed either shortly before sampling (e.g., the concentration of a solvent in the alveolar air or in blood during the work shift) or during the preceding day (e.g., the concentration of a solvent in alveolar air or in blood collected 16 h after the end of exposure) or during the last month when the chemical has a long biological halflife (e.g., the concentration of some metals in urine or in blood)(Lauwerys, Hoet, 1993). Internal dose may also mean the amount of chemical stored in one or in several body compartments or in the whole body. This usually applies to cumulative toxic chemicals. For example, the concentration of polychlorinated biphenyl (PCB) in blood is a reflection of the amount accumulated in the main sites of deposition (i.e., fatty tissues). Finally, with ideal biological monitoring tests, the internal dose means the amount of chemical bound to the critical sites of action. Such tests can be developed, for instance, when the critical sites are easily accessible (e.g., hemoglobin in case of exposure to carbon monoxide) or when the chemical interacts with a blood constituent in a similar way as with the critical target molecule (e.g., hemoglobin adducts) (Lauwerys, Hoet, 1993).

LEGACY BIOLOGICAL MONITORING

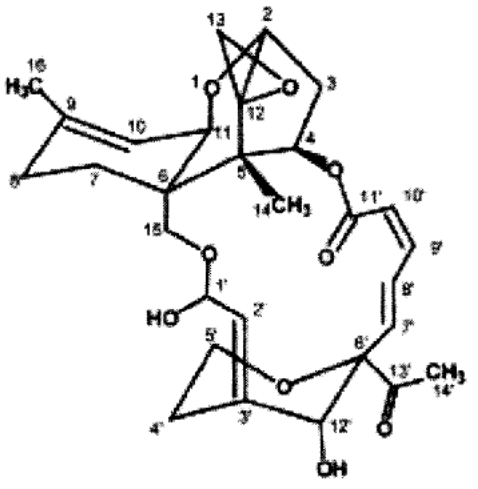
Another type of biological monitoring, which is similar to biological monitoring of effect, is legacy biological monitoring. Legacy biological monitoring, as defined here, is the measurement

of a specific determinant which contains none of the atoms of the molecule to which the organism was exposed. The most common example of this is an antibodies produced against small molecular weight molecules (Biagini et al. 1990). Some reactive small molecular weight molecules, while not immunogenic in their own right because of size and other limitations, may bind to constitutive polymers (such as host proteins) and become immunogenic, causing the production of specific antibodies. Alternatively or in addition, exposure to some small molecular weight proteins may cause the production of new antigenic determinants (NAD's) formed by interaction of these reactive small molecular weight compounds with selected protein carrier molecules. Antibodies can be made to these NAD's of constitutive proteins or to the parent hapten-conjugate (Biagini et al. 1988).

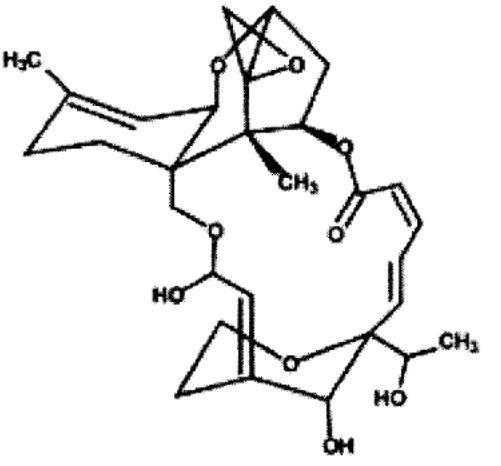
BIOLOGICAL MONITORING FOR TOXINS PRODUCED BY FUNGI

Numerous investigators have implicated *Stachybotrys* sp.and the toxins produced by them as being associated with adverse health effects (Johanning et al. 1996; Jarvis et al. 1998; Jarvis et al. 1995; Etzel et al. 1998; Hodgson et al. 1998). Of the toxins produced, the tricothecenes appear to be the most toxic in animal models (Nikulin et al. 1996). More than 60 tricothecenes produced by a variety of fungi, including *Fusarium, Trichoderma, Stachybotrys, Cephalosprium,* and *Tricothecium*. All have a double bond at C-9 and C-10 and an epoxy ring at C-12,13 and are thus called 12,13-epoxy-tricothecenes. The tricothecenes are grouped into four classifications (A, B, C, and D) based on chemical properties and the fungi that produce them(Ueno, 1985). Their general mechanism of action is inhibition of protein synthesis(Ueno, 1985). The satratoxins (Figure I) of *Stachybotrys* are macrocyclic in structure, and belong to group D. *Stachybotrys* also produces isosatratoxin G and S-isosatratoxin H in addition to at least nine immunosuppressant phenylspirodrimanes. All trichothecenes induce redness and irritation on the skin and can be considered irritants (Ueno, 1985). The metabolism of satratoxins has not been described in humans, but from the metabolism of similar macrocyclic tricothecenes, it probably involves a verrucarol (4-beta-15-hydroxy-12,13-epoxy-tricothec-9-ene) intermediate, formed by cleavage of the macrocyclic tricothecene ring at carbons 4 and 15 (Figure I), followed by further metabolism (Swanson et al. 1988) with the ultimate excretion of excretion of sulfate and glucuronide conjugates (Barel et al. 1994). Assays have been developed to measure tricothecene parent by immunoassay (Martlbauer et al. 1988; Dietrich et al. 1995). Direct methods for human urinary/sera biological monitoring of tricothecenes and/or their metabolites have not been described in the literature (with the exception of the work of Dietrich et al. in this volume). Indirect, non-specific methods, such as analyzing for antibodies directed against fungal antigens have been employed most commonly (Johanning et.al., 1996) for biological monitoring. These measurements, while not indicative of toxin exposure, do have use as indicators of exposure and possibly of effect. But in most cases, these measurements can be confounded by cross-reactivity and other systematic effects.

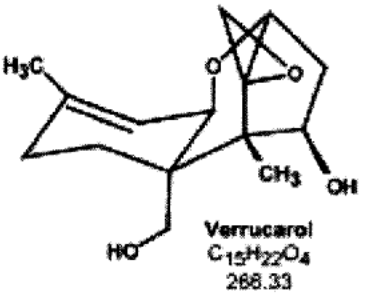
FIGURE I: Structures and molecular formulae of Satratoxins F and H and Verrucarol. Molecular weights are given below the formulae. Numbering system is shown for Satratoxin F.



Satratoxin F
$C_{29}H_{36}O_9$
526.59



Satratoxin H
$C_{29}H_{37}O_9$
529.60



Verrucarol
$C_{15}H_{22}O_4$
266.33

There is growing concern and an apparent increase in complaints about the adverse health effects of fungal bioaerosol exposure on occupants of waterdamaged buildings. Much is known concerning the allergenicity of fungi, however, mycotoxicosis (or direct toxicity from exposure to fungal metabolites) is an area where much recent attention and speculation has been directed. This is especially true of the fungus *Stachybotrys chartarum*. *S. chartarum* is a saprophytic fungus that is commonly found on cellulosic materials (wall paper, drywall) in high humidity or wet environments. *S. chartarum* can produce toxigenic macrocyclic tricothecene metabolites which have been shown to be potent protein synthesis inhibitors, immunosuppressive and highly toxic. Exposure to them has been implicated in cold and flu symptoms, sore throats, diarrhea, headaches, fatigue, dermatitis, psychological impairment, and pulmonary hemosiderosis in infants. At the present time there is no simple, reliable exposure indicator for macrocyclic tricothecene exposure. The halflife for excretion of these compounds is of the order of hours to days, and body burdens would be expected in the nanogram to picrogram range. These factors make traditional chemical analyses impractical or impossible at the pres-

ent time. The use of chemical and immunochemical methods in the search for a suspected common serum or urine metabolite has also been inconclusive. Therefore, indirect and non-specific tests including Tcell counts and proliferation, measurement of anti-fungal antibody levels and other simple hematological tests (CBC/Diff) have been used with limited success to attempt to associate exposure with clinical signs and symptoms.

As stated earlier, small chemicals can be immunogenic in their own right, especially if they bind to host proteins, are bound to proteins during exposure (such as fungal proteins) or damage host constitutive proteins as part of their toxic mechanism, possibly forming new antigenic determinants. There are many examples of this in the literature. Macrocyclic tricothecenes meet many of the outlined conditions for *de novo* immunogenesis, i.e., they are irritants, exposure is to combined protein/chemicals, and they damage constitutive proteins (endothelial cells). *De novo* biosynthesis of antibodies from mycotoxin exposure has been shown for aflatoxins (Autrup et al. 1990). In addition, Bulgarian investigators have implicated feed exposure to mycotoxins in cows in the production of anti-mycotoxin antibodies (Gelev et al. 1986). We have shown the measurement of *de novo* synthesized antibodies to be useful in evaluation of personal protective equipment in the ethical narcotics industry (Biagini et al. 1990). There exists the possibility that the toxins from *Stachybotrys* are immunogenic in their own right and that measurements of anti-toxin antibodies may be indicative of present and past exposure (legacy biomarkers). An almost sinister effect of the development of de-novo anti-satratoxin antibodies is false negative ELISA results. As the analyte would already be bound to antibody it would not be free to bind to the analytical anti-satratoxin antibody, yielding a negative result. We are actively investigating the possibility of anti-*Stachybotrys* toxin antibodies at the present time.

SUMMARY

Biological monitoring is the estimation of exposure to an agent through the measurement of biomarker(s) resulting from an internal dose of an agent. The biomarker(s) are typically the agent or its metabolite(s) in a biologically derived specimen. The biomarker also can be a highly specific, validated effect of the agent, such as elevated levels of zinc protoporphyrin in blood caused by exposure to lead. Due to advances in molecular biology, genetics, analytical chemistry, and other basic sciences, it is now possible to detect smaller amounts of analytes and contaminants and smaller biological changes, as well as to identify mechanisms at the cellular and molecular levels. From these advances, in some cases, a molecular continuum from exposure through disease may be constructed. Numerous investigators have implicated putative exposure to macrocyclic tricothecenes and other compounds from *Stachybotrys chartarum* as being associated with a plethora of signs and symptoms of disease. To date, no compelling biological marker evidence for exposure to *Stachybotrys chartarum* mycotoxins or other metabolites has been presented. There is no doubt that exposure to *Stachybotrys chartarum* is associated with an increased prevalence of self-reported symptoms. However, evidence of the association of these complaints with frank disease is lacking. More biological monitoring research is needed solidify these associations, if present.

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COMPARATIVE STUDIES OF FUNGAL MEDIA FOR THE RECOVERY OF STACHYBOTRYS CHARTARUM FROM ENVIRONMENTAL SAMPLES

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ABSTRACT

Five commonly used fungal media were used to determine the relative recovery efficiencies of *Stachybotrys chartarum (SC)* by culturing sixty-five unknown environmental samples from building materials. These fungal media were cornmeal agar (CMA), Czapek cellulose agar (CCA), 2% malt extract agar (2% MEA), 1% malt extract agar (1% MEA) and rose bengal agar (RBA). The samples were first examined microscopically for the presence of *SC*. It was found that all five fungal media were all suitable for the recovery of *SC* from the environmental samples. The recovery frequency of *SC* from bulk samples ranged from 87.7% on 2% MEA to 95.4% on CMA. Qualitative differences of colony growth and interaction between *S. chartarum* and other fungi were observed on the media. CMA yielded the best sporulation and highest recovery rate of *SC* from the environmental samples. The other four media also supported the isolation and recovery of *SC* but at reduced rates.

KEY WORDS:

Stachybotrys chartarum, fungal contamination, media selection.

INTRODUCTION

Stachybotrys chartarum (Ehrenb.: Link) Hughes (syn. *S. atra*) is a cosmopolitan fungus and has been isolated from paper, seeds, soil, textiles, and dead plants (Ellis, 1971). *Stachybotrys chartarum* (*SC*) is a soft rot fungus and grows well on wet delignified cellulose-containing materials such as straw or paper. It has found a niche in the indoor environment, where cellulose-containing materials are abundant. It has frequently been observed growing on water-damaged drywall, wall paper, ceiling tiles of high cellulose content, and cellulose fiber-based carpets. In addition to cellulose, it is known to utilize many sugars, organic acids, nitrogen salts and amino acids, starch, chitin and wool, and cellulose derivatives (such as methyl cellulose and cellophane). It is apparently an adaptable fungus (Domsch et al. 1980, 1993).

SC and species of *Aspergillus*, and *Penicillium* have been found to be common in buildings with known indoor air and building-related problems and illnesses (Croft et al. 1986; Flannigan et al. 1991; Johanning et al. 1993; Hodgson et al. 1998). Johanning (1995) and his colleagues (1993, 1995 and 1996) reported additional cases of office building-associated illnesses attrib-

uted to *SC*. Several cases of infant hemosiderosis have been linked to *SC* exposure (CDC, 1996; Etzel et al. 1998).

Cellulose-enriched media are frequently used to isolate *SC*. The AIHA Field Guide for the Determination of Biological Contaminants in Environmental Samples (Dillion et al. 1996) suggests cellulose agar (CA) as an appropriate medium for *Stachybotrys* isolation. Jong and Davis (1976) studied the related genera *Stachybotrys* and *Memnoniella* based on growth and morphology of fungal colonies on cornmeal agar (CMA). Other media have been used to isolate *Stachybotrys* from the environment including Czapek cellulose agar (CCA), 2% malt extract agar (2% MEA) and rose bengal agar (RBA).

This study is to determine the relative recovery efficiencies of *S. chartarum* and to evaluate growth and spore production of the fungus from five commonly used fungal media by comparing recovery from actual unknown environmental samples. A preliminary report of this study was presented at the AIHACE in Atlanta, 1998 (Tsai et. al, 1998).

MATERIALS AND METHODS

The media used for inoculation were 2% malt extract agar (20g Difco malt extract broth and 20g agar in 1000 ml distilled water; 2% MEA), 1% malt extract agar (10g Difco malt extract broth and 20g agar in 1000 ml distilled water; 1% MEA), cornmeal agar (Difco; CMA), rose bengal agar (Difco; RBA), and Czapek cellulose agar (35 g Difco Czapek-Dox powder, 10 g Sigma cellulose powder and 20 g agar in 1000 ml distilled water; CCA). Environmental samples found by microscopic examination to have *Stachybotrys chartarum* contamination were used for this study. A sample of approximately 0.1 to 0.2 g was weighed, extracted and suspended in sterile distilled water, diluted and inoculated at 100x, 4,100x and 16,400x, spread plates and incubated at 25°C. All plates were examined and characterized in seven to ten days. A total of sixty-five samples were analyzed.

All data collected were subject to descriptive statistical analysis using Microsoft Excel Analysis Toolpak. The *SC* concentration of each sample was calculated and presented as CFU/g. In order to get the recovery ratio of *SC*, we sorted out the agar medium with the maximum concentration of each sample. The recovery ratio is defined as agar medium concentration divided by maximum agar medium concentration of each sample. We describe the recovery ratio distribution of each medium using five-number summary (minimum, quartile Q1, median, quartile Q3 and maximum) and mean.

RESULTS AND DISCUSSION

Sixty-five bulk samples from building materials were examined microscopically for the presence of *S. chartarum*. All five fungal media were suitable for the recovery of *SC* from the bulk samples. The recovery frequency of *S. chartarum* from bulk samples ranged from 87.7 % (57 of 65 samples) on 2% MEA to 95.4 % (62 of 65 samples) on CMA (Table I). The highest absolute recovery of *SC* was on CMA but there were no significant differences between CMA and the other four media used (χ^2=2.97 < $\chi^2_{0.95(4)}$=9.488). Because the number of culturable spores

was unknown, the medium with the highest colony count for each sample was considered to have a recovery ratio of 1. The percentage of samples with a recovery ratio of 1 occurring ranged from 4.6 % (3 of 65 samples) on RBA to 67.7 % (44 of 65 samples) on CMA (Table II). CMA, 1% MEA, RBA, 2% MEA, and CCA had mean recovery ratios of 0.86 ± 0.07, 0.63 ± 0.10, 0.55 ± 0.09, 0.54 ± 0.12 and 0.53 ± 0.11, respectively (Table III).

TABLE I: Recovery rate of *S. chartarum* from environmental bulk samples.

Media	SC Detected	Total samples	Recovery	Rank
CMA	62	65	95.4%	1
1% MEA	60	65	92.3%	2
RBA	60	65	92.3%	2
CCA	58	65	89.2%	4
2% MEA	57	65	87.7%	5

TABLE II: The frequency of highest recovery of *Stachybotrys chartarum* occurring in each medium from environmental bulk samples.

Media	Samples w/ highest recovery	Total samples	Percentage of highest recovery	Rank
CMA	44	65	67.7%	1
CCA	9	65	13.8%	2
1% MEA	9	65	13.8%	2
2% MEA	4	65	6.2%	4
RBA	3	65	4.6%	5

TABLE III: Summary of recovery ratio of *S. chartarum* from 65 bulk samples. The medium with the highest average colony count for each sample was considered to have a recovery ratio of 1.

	2% MEA	1% MEA	RBA	CCA	CMA
Minimum	0.00	0.00	0.00	0.00	0.00
Q1	0.23	0.47	0.40	0.28	0.88
Mean	0.54	0.63	0.55	0.53	0.86
Median	0.68	0.70	0.56	0.54	1.00
Q3	0.84	0.89	0.76	0.81	1.00
Maximum	1.00	1.00	1.00	1.00	1.00

Qualitative differences of colony growth and interference between *SC* and other fungi were observed on these media. Colony development and sporulation were generally typical on CMA and RBA whether other fungi were present or not. Colony development and sporulation were often interfered with and retarded on 1% MEA, 2% MEA, and CCA when other environmental fungi, particularly *Aspergillus*, *Chaetomium*, *Cladosporium*, and/or *Penicillium* species were present. A stereomicroscope was often necessary to detect and count colonies of *SC* in the presence of interfering fungi. Similar interference by different species of *Penicillium* was reported by Gravesen et al. (1994).

CONCLUSIONS

CMA yielded the best recovery rate and good sporulation of *SC* from sixty-five environmental samples. The other four media also supported the isolation and recovery of *SC* but at reduced rates. On the basis of this study, it is suggested that CMA should be routinely used for environmental sampling, whether air or bulk, when *SC* is suspected.

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HETERODUPLEX DNA FINGERPRINTING OF PENICILLIUM BREVICOMPACTUM FROM HOUSE DUST

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ABSTRACT

It is widely believed that many osmotolerant microfungi, including toxigenic species of *Aspergillus* and *Penicillium* proliferate on indoor substrata such as dust and broadloom even in the absence of appreciable moisture. This hypothesis has been offered as an explanation for the disproportionate abundance of propagules of these species in indoor environments, relative to their representation in outdoor reservoirs such as air and soil.

We obtained several thousand isolates of *Penicillium* from 367 homes in southwestern Ontario, Canada. From these, 75 isolates of *P. brevicompactum* representing 54 houses were selected based on micromorphological and physiological uniformity. Sequences of PCR-amplification products of polymorphic genetic loci were compared between isolates using heteroduplex mobility assay (HMA), demonstrating two primary, genetically divergent groups which appear to be stable, clonally-reproduced lineages within the dust mycoflora. These clones are distributed throughout the sample population and co-exist at several sites, suggesting that in absence of objective moisture problems, the principal amplifiers of *P. brevicompactum* lie outside the building environment. It is possible that mechanical / filtration effects (e.g. differential removal of propagules by vacuum cleaning and elutriation in air conveyance systems), and the relatively long spore viabilities of trichocomaceous anamorphs play a significant role in concentrating propagules of these microfungi in homes.

KEY WORDS

Penicillium brevicompactum, population, house dust, indoor air quality, heteroduplex mobility assay

INTRODUCTION

It is widely agreed that the outdoor environment contributes substantially to the burden of biological particulate present in building interiors (Miller, 1992). Despite that airborne fungal spores of outdoor origin are accepted to be amongst the principal allergenic mediators of seasonal allergic rhinitis, the presence of amplifiers of fungi within the building envelope presents a more serious concern. This is so for two main reasons: 1) In contrast to most outdoor surfaces, building materials of organic origin remain devoid of active growth until they become

water damaged, at which point the absence of microbial competition facilitates the unbridled growth of primary fungal colonists; and 2) the indoor environment contains a finite dilution volume of air, permitting the development of airborne concentrations of these contaminants which profoundly exceed outdoor levels. In the case of persistently wet drywall, the species most often observed are *Aspergillus nidulans* group, *A. versicolor, Chaetomium spp., Penicillium brevicompactum* and *Stachybotrys chartarum*. Curiously however, certain of these species, notably *A. versicolor, P. brevicompactum* and *P. chrysogenum* may be observed in abundance even in building environments lacking conspicuous amplifiers. Investigations of both outdoor air and organic substrata fail to a demonstrate sufficient presence of propagules of these fungi to account for their predominance in the indoor spora relative to other typical background taxa, such as phylloplane moulds. Thus, the culture-presence of these taxa from dust isolations, many of which range from xerotolerant to frankly xerophilic, has been interpreted as evidence for the proliferation of these species in the dust substrate under "normal" conditions (e.g. low water activity) (e.g. Davies, 1960; van Bronswijk, 1981). Despite considerable observation and documentation of these species from household dust and indoor air, there is scant empirical evidence to support this widespread hypothesis of generalized cryptic indoor proliferation. Through the use of molecular genetic fingerprinting methods, we have sought to address this knowledge gap.

In cooperation with the Canada Mortgage and Housing Corporation (CMHC) we have examined populations of P. brevicompactum obtained from a large sample of housing in southern Ontario using heteroduplex mobility analysis of PCR-amplified polymorphic genetic loci. In this method, homologous PCR-amplicons from different isolates are combined in a pair-wise fashion, heat-denatured and allowed to reanneal. Differences in base sequence between the two homologous amplicons are revealed by the differentially slower electrophoretic migration of double-stranded DNA hybrids containing base mismatches compared to reannealed matching amplicons. This technique has been used successfully in the screening of human genes for mutations of medical significance (e.g. White et al. 1992), and in the epidemiological investigation of human viral populations (Delwart et al. 1993; 1994). We present preliminary results of these investigations.

MATERIALS AND METHODS

Collection and characterization of isolates:

Vacuum cleaner bag samples of carpet dust were collected from 369 houses in Wallaceburg, Ontario, Canada by a private company under contract from Canada Mortgage and Housing Corporation over a period of five months starting in January, 1994. Fungal isolates were obtained using a standard dilution plating technique (Malloch, 1981). Isolates of *Penicillium* were identified using conventional methods (e.g. Pitt, 1979). Isolates selected for genetic screening were indistinguishable based upon microscopic morphology and physiological profiles on standard growth media (Pitt, 1979).

Isolation of high molecular weight DNA:

Small-scale preparations of high molecular weight total DNA were obtained using the method described by Scott et al. (in press), in which conidia were harvested in 95% ethanol from Petri-plate cultures, dried and ground using acid cleaned diatomaceous earth. DNA was isolated in a lysis buffer containing 1.4 M NaCl, 2 % w/v CTAB, 200 mM Tris(HCl pH 8.0 and 20 mM EDTA (adapted from Weising et al. 1995) at 65°C, and extracted twice with chloroform:isoamyl alcohol (24:1) and precipitated with 100 % isopropanol for 10 min at - 80°C. The pellets were rinsed with 70 % ethanol, dried and resuspended in 200 µL Tris-EDTA (10 mM and 1 mM, respectively) (TE) pH 8.0. DNA was treated with Ribonuclease A at a concentration of 0.2 µg/µL, incubated for 30 min at 37°C, extracted with chloroform:isoamyl alcohol as above, and precipitated with an equal volume of 100 % ethanol at -80 °C following the addition of sodium acetate to a concentration of 0.3 M. DNA solutions were pelleted, rinsed with 70 % ethanol, dried and resuspended in TE at pH 8.0 at a standard concentration determined spectrophotometrically.

Amplification of polymorphic markers:

Three polymorphic loci consisting of introns in the genes encoding beta-tubulin (BenA), histone 4 (H4) and the region spanning the internal transcribed spacers (ITS1 and -2) of ribosomal DNA (rDNA) were PCR-amplified using the primer sequences given in Table I. PCRs comprised 1 unit of Taq DNA polymerase (Boeringher Mannheim), 50 mM KCl, 2.0 mM MgCl2, 250 µM each of dATP, dTTP, dCTP and dGTP, 0.2 mM of each primer and approximately 60 ng high molecular weight template DNA in 1 x Promega Taq DNA polymerase buffer in a total reaction volume of 50 µm overlaid with a drop of sterile mineral oil to prevent evaporation. A template-free reaction was included in each set of reactions. Reactions were carried out in a PTC-100 thermocycler (MJ Research). The typical PCR profile used consisted of 94°C for 30 s to denature, 58°C for 30 s to anneal primers and 72°C for 30 s to extend. This profile was repeated for 30 cycles, followed by a final extension at 72°C for 2 min. PCR yield was roughly quantified based on ethidium bromide staining and UV visualization following electrophoresis on 1.2% agarose gels.

TABLE I: Oligonucleotide primers.

Locus	Primer Name	Source	Primer sequence (5'-3')
BenA	Bt2a	Glass & Donaldson, 1995	GGT AAC CAA ATC GGT GCT GCT TTC
	Bt2b	Glass & Donaldson, 1995	ACC CTC AGT GTA GTG ACC CTT GGC
H4	H41a	Glass & Donaldson, 1995	GCT ATC CGC CGT CTC GCT
	H41b	Glass & Donaldson, 1995	GGT ACG GCC CTG GCG CTT
rDNA ITS1-2	ITS5	White et al. 1990	GGA AGT AAA AGT CGT AAC AAG G
	WNL1	Untereiner et al. 1995	TAT GCT TAA GTT CAG CGG

Heteroduplex mobility assay (HMA):

For each locus examined, PCRs were diluted to 50 % of the original concentration, with 4 mM EDTA and 50 mM KCl and combined in equimolar proportion in a total volume of 10 µL,

and overlaid with a drop of sterile mineral oil. PCRs of sequentially numbered isolates were combined in an pair-wise manner such that each numerically adjacent pair were combined with each-other, and the fist and last isolate of each series were combined. Reactions were heated to boiling for 2 min and immediately annealed at 65°C for 2 min. Based upon the results of this first pass, all adjacent "like" isolates were reduced to a single representative strain by transitive property. Subsequent rounds of HMA compared non- numerically juxtaposed "proxy" isolates ultimately reducing the entire population to a set of genotypically distinct alleles represented by a minimum number of proxy isolates for each locus tested.

GEL PREPARATION, ELECTROPHORESIS AND IMAGING:

A solution of 12.0 % acrylamide, 0.2 % bisacrylamide and 0.04 % ammonium persulfate was prepared in 1 x TBE cast in a BioRad Protean electrophoresis apparatus to which 1.2 % agarose and 0.2 % N,N,N',N'-tetramethylethylenediamine (TEMED) were added after degassing of the solution and immediately prior to casting. Gels were cast in a BioRad Protean electrophoresis apparatus, cooled to 12°C using a constant temperature refrigerated water bath and electrophoresed at 10 V/cm for up to 20 hr. Following electrophoresis, gels were stained in 250 ng/mL ethidium bromide for 2 hr, destained in distilled water for 3-4 hr and visualised on an ultraviolet light transilluminator at 300 nm (Fotodyne).

RESULTS AND DISCUSSION

Overall, studies of the internal transcribed spacer region of nuclear ribosomal DNA (rDNA), as well as introns in the beta-tubulin and histone 4 genes suggest that domicile isolates of *P. brevicompactum* are highly genetically homogeneous as compared to geographically unique isolates (data not shown). Notwithstanding this observation, however, multiple genotypes of *P. brevicompactum* were isolated commonly within individual houses. By cloning and sequencing genetically different strains, we were able to show that sequences of introns in the (-tubulin gene differed in substantial ways, negating the possibility that these strains arose spontaneously, in situ, from a common ancestor as a result of simple mutation (Scott et al. in press). In *P. brevicompactum*, genotypic differences observed in the β-tubulin gene tended to correspond to differences in the histone 4 gene, supporting the complex nature of these strain differences. Analysis of geographically unique "natural" isolates of *P. brevicompactum* showed these strains to be highly distinct compared to all dust-borne Wallaceburg strains, supporting the existence of true "domestic" strains with geographically local distribution throughout the Wallaceburg area.

A preliminary set of multilocus fingerprints is presented in Table II. The lowest degree of allelic variation was absent in the histone 4 locus (2 alleles) whereas the highest variation (6 alleles) was seen in the beta-tubulin locus (see Table III). The most commonly observed multilocus fingerprints accounted for 52 and 24 % of the isolates characterized, respectively (see Table IV). The existence of three multilocus genotypes, CAA', CAB and AAB at roughly 5, 10 and 7 % of the population, respectively implies that the population is not strictly clonal. Alleles comprising the second most commonly observed fingerprint (BBC, Table IV) constituted a

bona fide linkage group which appears to be completely divergent from the multilocus allelic motifs found in the primary group.

TABLE II: Multilocus genotypes for *P. brevicompactum* isolates.

House Number	Isolate number	Good / Bad	Bt2	H41	ITS	House Number	Isolate number	Good / Bad	Bt2	H41	ITS
42			A	A	A	132	2	BAD	B	B	C
45	1	BAD	B	B	C	136		BAD	A	A	A
45	2	BAD	B	B	C	165			A	A	A
62			C	A	A	166			A	A	A
65	1		A	A	A	170			A	A	A
65	3		A	A	A	183				A	A
65	4		A	A	A	185			A	A	A
65	5		B	B	C	189			A	A	A
65	6		A	A	B	192	1		A	A	A
67	1		D			192	2		A	A	A
67	2			A	A	201		BAD	A	A	A
67	3			A	A	204				A	B
67	4			A	A	217			A	A	A
67	5		A	A	B	228			C	A	B
67	6		B	B	C	233	1		B	B	C
67	7			A	A	233	2		C		B
70				A	A	240			E		A
72				A	A	244	1		F		B
74				A	A	244	2		A		A
75	1		C	A	A	245			A		A
75	2		C	A	B	251					B
75	3		C	A	D	259			A		A
79			C	A	A	263			A		A
81		GD		A	A	264			E		A
87			C	A		266			A		A
91			B	B	C	273			A		A
98			A	A	A	274		GD	A		A
99			A	A	A	280			A		A
100		BAD		A	A	306			A		B
109	1	GD	A	A	B	319			A		A
109	2	GD	A	A	A	322			A		A
112			A	A	B	325			C		B
114			A	A	B	340			B	B	C
117		BAD	A	A	A	353			B	B	C
119	1		A	A	A	373			A		A
119	2		A	A	A	374			C		B
132	1	BAD	B	B	C						

TABLE III: Synopsis of genetic loci characterized.

Locus	Isolates characterized	Number of houses (with multiple isolates)	Unique sequence motifs observed
Bt2	62	46 (10)	6
H41	53	34 (9)	2
ITS	71	53 (10)	4
Combined loci:	**42**	**29 (9)**	**6**

Although the BBC type occurs with less frequency than AAA, the occurrence of BBC in bad houses is disproportionately high (i.e. ratio of bad:total houses, Table IV). However, two thirds of houses from which multiple isolates of *P. brevicompactum* were obtained showed mixed populations of two-or more multilocus genotypes suggesting that clonal dominance within houses is likely not a generalized phenomenon. Furthermore, this observation does not support the proliferation of this species in the absence of conspicuous moisture problems.

TABLE IV: Comparative distribution of principal multilocus genotypes (%).

Multilocus type (Bt2, H41, ITS)	Proportion of fully characterized isolates	Presence in all houses	Presence in bad houses	Ratio of bad houses to total houses
A, A, A	52	62	10	14
A, A, B	7	10	-	-
B, B, C	24	28	7	25
C, A, A	5	7	-	-
C, A, B	10	14	-	-
C, A, D	2	3	-	-

Other factors besides proliferation in situ may lead to accumulation of viable *Penicillium* spores in dust in homes absent of indicators of mould problems (e.g. in good repair, clean and free from water damage) where objective measurements demonstrate a high standard of indoor air quality. This study was intended to demonstrate the natural sources of dust-borne propagules of *P. brevicompactum*. We have observed *P. brevicompactum* on numerous occasions growing on water damaged indoor substrates, particularly drywall. However, despite the low water activity at which this species is capable of growth (Aw = 0.78 at 25°C; Samson et al. 1997), we have not observed conidiogenous apparati of *P. brevicompactum* in microscopic preparations of broadloom materials, except where moisture problems have been observed (e.g. flooding, carpeting located in a basement, &c.). This species occurs naturally on a number of substrates outdoors including decomposing mushroom fruiting bodies and other organic matter; however, it is poorly represented in outdoor air samples. It has been our experience that this species is isolated from outdoor air, albeit infrequently. Thus it is feasible that dilute levels of spores of this taxon might enter houses from outdoors and simply accumulate in dust, either due to differentially long viability period of spores of these species or through mechanical means.

To address this question, we examined the longevity of dust-borne fungal propagules under dry storage conditions in samples of dust from selected Wallaceburg houses over a three year period. Results indicated that spores of species of *Aspergillus* and *Penicillium* remain viable for a much longer period of time as compared to most phylloplane fungi under our storage conditions (e.g. *Alternaria, Cladosporium, Epicoccum and Ulocladium*). These results are in contrast to [1] Allelic types of Bt2, H41 and ITS, respectively.
Kramer and Pady (1968), but agree with the observations of Sussman (1968). Thus, the culture-presence of high numbers of *Aspergillus* and *Penicillium* relative to phylloplane taxa from household dust may be an artifact of the longer period of spore viability of the former group relative to the latter.

We hypothesize that various routine mechanical processes within houses permit the accumulation of fungal spores. Since spores of *Aspergillus* and *Penicillium* are typically in the size range of 2-5 (m in diameter it is possible (and widely conjectured), that these particles may penetrate inefficient filters, such as dust bags in portable vacuum cleaners or filters in forced-air heating systems, and thus, not be efficiently removed from the indoor environment by these processes. This being the case, a properly installed, externally-vented central vacuum cleaner may help to reduce the accumulation of indoor mould spores.

Inefficient removal of fungal propagules coupled with the longevity of these spore types might account, in part, for the observed "populations" of these fungi in household dust. As well, materials in houses, such as broadloom, may function as long-term accumulators of fungal spores, and constitute rich, passive reservoirs of indoor fungi.

CONCLUSIONS

Although these results support a general allochthonous source of *P. brevicompactum* spora in the absence of moisture problems, given the present data-set, it is not possible to negate a generalized, low-grade contribution by *in situ* amplification. Based on our results, we hypothesize two general cases:

1) Where the presence of moisture problems facilitates the amplification of *P. brevicompactum* indoors, a strong clonal signal can be expected. Our observations from many building inspections show a strong albeit anecdotal correlation between observed presence of fungi on surfaces (e.g. basement walls) and concentrations revealed by indoor air samples elsewhere in the building.
2) In the absence of moisture accumulation indoors, elevated levels of *P. brevicompactum* indoors (e.g. in dust) disproportionate to the abundance of this species in the outdoor air are likely due to filtration effects.

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THE TRICHODIENE SYNTHASE GENE FROM *STACHYBOTRYS CHARTARUM*: A POTENTIAL DIAGNOSTIC INDICATOR OF INDOOR CONTAMINATION

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ABSTRACT

The hyphomycete *Stachybotrys chartarum* readily grows on damp cellulosic areas in buildings producing spores that may contain highly toxic trichothecenes. Here we report the cloning, sequencing of the gene for trichodiene synthase which is the first enzyme of the pathway uniquely dedicated to the synthesis of trichothecenes. The predicted amino acid sequence shows regions of high conservation. Nucleotide sequence divergence permits the selection of PCR primers that can detect the trichodiene synthase gene of *S. chartarum* in DNA diagnostic strategies.

KEY WORDS

Stachybotrys chartarum, trichodiene synthase gene, DNA diagnostics

INTRODUCTION

Stachybotrys chartarum (=*S. atra*) is a toxigenic fungus that grows readily on water-soaked cellulosic building materials such as drywall, waferboard and wood. Because its spores may contain highly toxic macrocyclic trichothecenes it is one of the more dangerous causal agents of "sick building syndrome". Mounting evidence has implicated this fungus as the causative agent of sudden infant death from acute pulmonary haemosiderosis (CDC, 1995). Adults exposed to office environments manifesting growth of *S. chartarum* may show symptoms suggesting "immune competency dysfunction" (Johanning et al. 1996). In fact, the accumulating body of evidence indicates that inhalation exposure to spore bound mycotoxins may play an important role in compromising human health in environments ranging from mines (Venitt & Biggs,1994; Sram et al. 1993) to libraries (Hay,1995).

The trichothecenes comprise a complex family of sesquiterpene epoxides that are potent inhibitors of protein biosynthesis in eukaryotes. The first step in the production of these mycotoxins is the synthesis of trichodiene through the cyclization of trans, trans-farnesyl PPi by trichodiene synthase (TS). The gene for this enzyme has now been cloned and sequenced for several crop pathogens and agents of post-harvest spoilage in the ascomycete genus *Gibberella* (Genbank, #U22464 and #M64348) and its *Fusarium* anamorphs (Genbank, #M27246 and #U15658), and for *Myrothecium roridum* (Genbank #AF009416). Here we report the cloning and characterization of the TS gene from *S. chartarum*. The judicious selection of sequences specific to the *S. chartarum* gene together with the development of appropriately stringent PCR conditions have permitted the specific amplification of this *Stachybotrys* gene.

These results indicate that PCR could be used as a diagnostic indicator of *Stachybotrys* in air samples from infected buildings.

MATERIALS AND METHODS

FUNGAL ISOLATES IN THIS STUDY

Species:	Strain/source:
Fusarium sporotrichioides	NRRL 3299 USDA
Myrothecium cinctum	Indoor air sample, Ont. Canada
Myrothecium roridum	ATCC 52485
Memnoniella sp.	Indoor air sample, Ont. Canada
Stachybotrys chartarum	MUCL 40562, Indoor air sample, Ont. Canada
Trichoderma viride	University of Toronto Collection

DNA isolation: The fungus was grown on a modified Leonian's liquid medium (Malloch, 1981). Mycelial balls were harvested by filtration; then rinsed with distilled water. The drained-dry mycelium was ground to a powder in liquid nitrogen. Two volumes of lysing buffer containing 100 mM Tris(HCl pH8, 20mM EDTA pH8, 1.4M NaCl and 2% (w/v) Cetyltrimethylammonium bromide (CTAB) was added and incubated at 60ªC for 1 hr. Room temperature lysate was deproteinized twice with an equal volume of chloroform-isoamyl alcohol (24:1). DNA was precipitated with 2/3 volume of cold isopropanol. The pellet was rinsed with 70% (v/v) ethanol, dried and dissolved in 2 ml of TE (10mM Tris (HCL, 1mM EDTA pH8). After RNase A digestion (50 µg /ml at 37ªC for 30 min). The mixture was deproteinized sequentially with equal volumes of Tris(HCl buffered phenol, phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). DNA was strand precipitated by adding 0.1 volume of 3 M sodium acetate and 2 volumes of -20ªC ethanol, rinsed in 70% (v/v) ethanol, dried and resuspended in 1 ml of TE.

CLONING OF THE TRICHODIENE SYNTHASE GENE FROM STACHYBOTRYS CHARTARUM.

Two oligonucleotide primers were (nucleotides 480-514 and 1302-1268) were used to make non-radioactive DNA probe for a conserved 823 bp region of the trichodiene synthase (TS) gene using a PCR DIG Probe Synthesis kit (Boehringer Mannheim) and total *F. sporotrichioides* DNA.

S. chartarum DNA, 10 µg each, was digested separately with BamH I, EcoR I, Hind III and Pst I, electrophoresed in a 0.7% agarose gel and Southern blot transferred in 20xSSC to a nylon membrane (Boehringer Mannheim). A standard buffer of 5xSSC, 0.1%(w/v) N-lauroylsarcosine, 0.02%(w/v) SDS and 1% blocking reagent was used for pre-hybridization and hybridization solutions. The membrane was pre-hybridized for 2-3 hr at 57ªC. Denatured probe (1 µ/ml) was added to the incubation container. After hybridization overnight at 57ªC, the membrane was washed twice for 15 min in 2x SSC, 0.1% SDS at room temperature, followed by two 15 min washes in 0.5x SSC, 0.1% SDS at 56ªC. Signals from hybridized probe were detected using the DIG Luminescent Detection kit (Boehringer Mannheim). A strong signal was detected at a fragment size of about 4.3Kb for the *Pst* I digested DNA. *Pst* I digested DNA

fragments of around 4.3 Kb size were purified from agarose gel slice, ligated to pGEM3Zf(+) and transformed into E. coli DH5α competent cells prepared according to Hannahan (1985). Positive transformants were detected by DNA hybridization to the non-radioactive TS DNA probe from *F. sporotrichioides*.

RT-PCR USING S. CHARTARUM RNA

Total RNA was isolated from 20mg of mycelium, using Glass MAX spin Cartridge System (Gibco/BRL). Two 1μg aliquots of RNA were treated with the amplication grade DNase I to remove residual DNA. One aliquot was used as template to synthesize first strand cDNA. A 20μl mix of RNA, 0.5 μM anti sense primer, 10 mM DTT, 500 μM dNTPs and RT buffer was incubated at 42°C for 2 min. 200 units of Superscriptπ reverse transcriptase (RT) (Gibco/BRl) was added and incubation continued for 50 minutes. The reaction was heated at 70°C for 15 min to inactivate RT. The other aliquot was used for positive control where 1μl of DEPC treated water replaced RT. 10μl of each reaction was subjected to PCR, using both the sense and antisense primers.

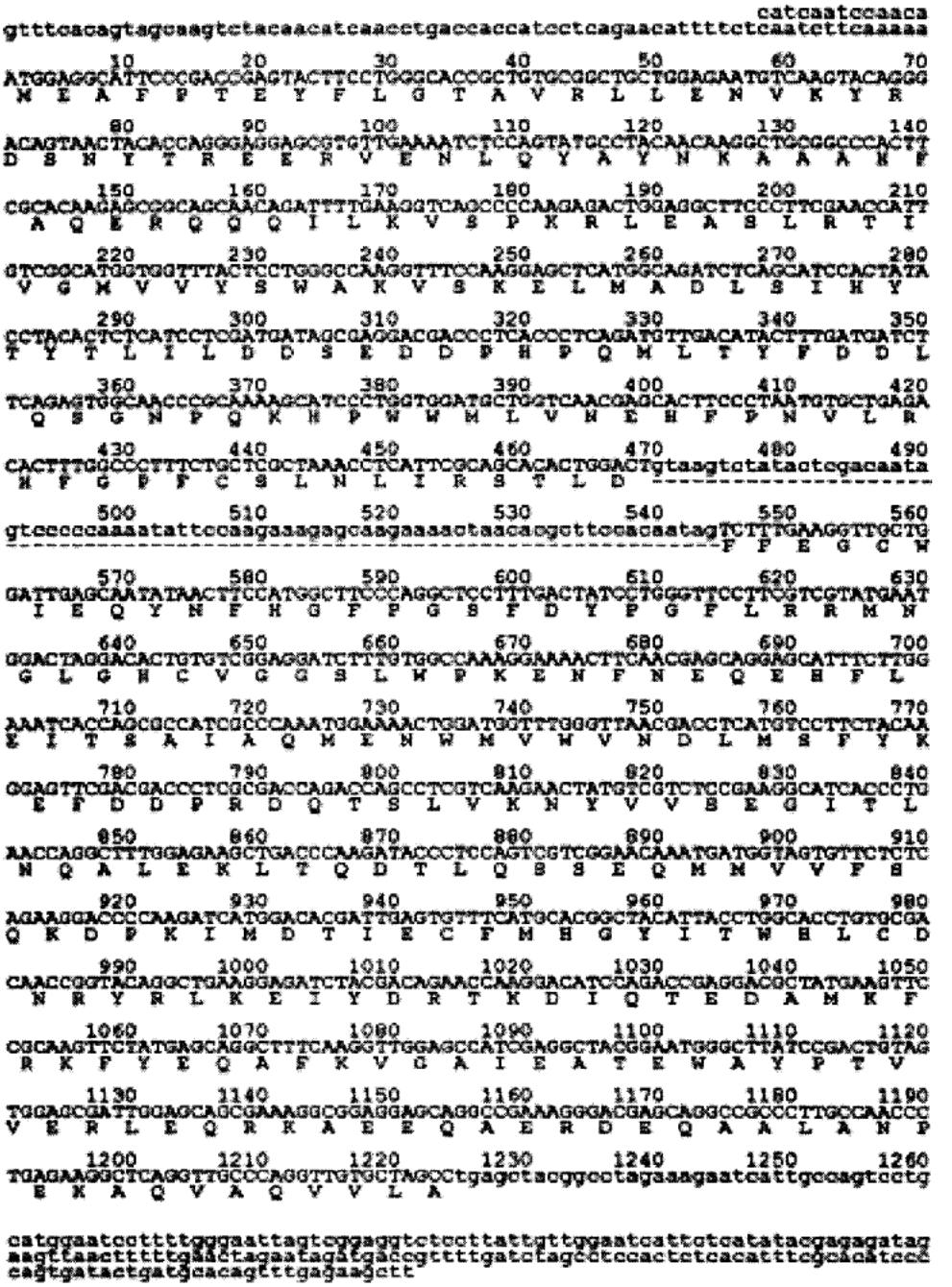
RESULTS

By comparing the DNA sequences of trichodiene synthase (TS) from four different fungal species, *Fusarium poae* (Genbank, #U15653), *F. sporotrichioides* (Genbank, #M27246), *Gibberella pulicaris* (Genbank, #M64348) and *G. zeae* (Genbank, #U22464) we selected two PCR primers that bordered a highly conserved DNA sequence region in all four species. Using these primers a DIG-labeled DNA probe for the TS gene was generated by PCR using total DNA from *F. sporotrichioides* as the template. The identity of the probe was confirmed by cloning and sequencing. This probe was used to identify DNA fragments that contained the TS gene in Southern blot experiments. When hybridized to *Bam*H I, *Eco*R I, *Hind* III and *Pst* I digested DNAs at 57°C, the probe gave a clear single band signal for each enzyme: *Bam*H I, 18Kb; *Eco*R I, 5.4Kb; *Hind* III, 2.8Kb; *Pst* I 4.3Kb.

The *Pst* I fragment containing the TS gene was cloned and the resulting clone was subcloned as a 2.8 Kb *Pst* I/*Hind* III fragment. This fragment was sequenced and the TS gene was identified from an amino acid sequence analysis of all six reading frames. Independent confirmation of the DNA sequence was obtained by cycle sequencing both strands of the PCR product generated using primers outside the TS gene. Figure I contains the sequence of the TS gene from *S. chartarum* (GenBank Accession Number AF053926).

To determine the presence and position of introns RT-PCR was used to generate cDNA from total *S. chartarum* RNA. Gel electrophoresis of the cDNA PCR product and the corresponding genomic PCR product indicated that the cDNA product was smaller. The cDNA PCR product was cleaned with glass milk and cycled sequenced using an ABI sequencer. The sequence for the cDNA corresponded to that of the genomic sequence but lacked sequence at one site indicating the presence of a single intron of 75 nucleotides. Figure I indicates the position of the intron in the gene and contains the predicted amino acid sequence of TS from *S. chartarum*

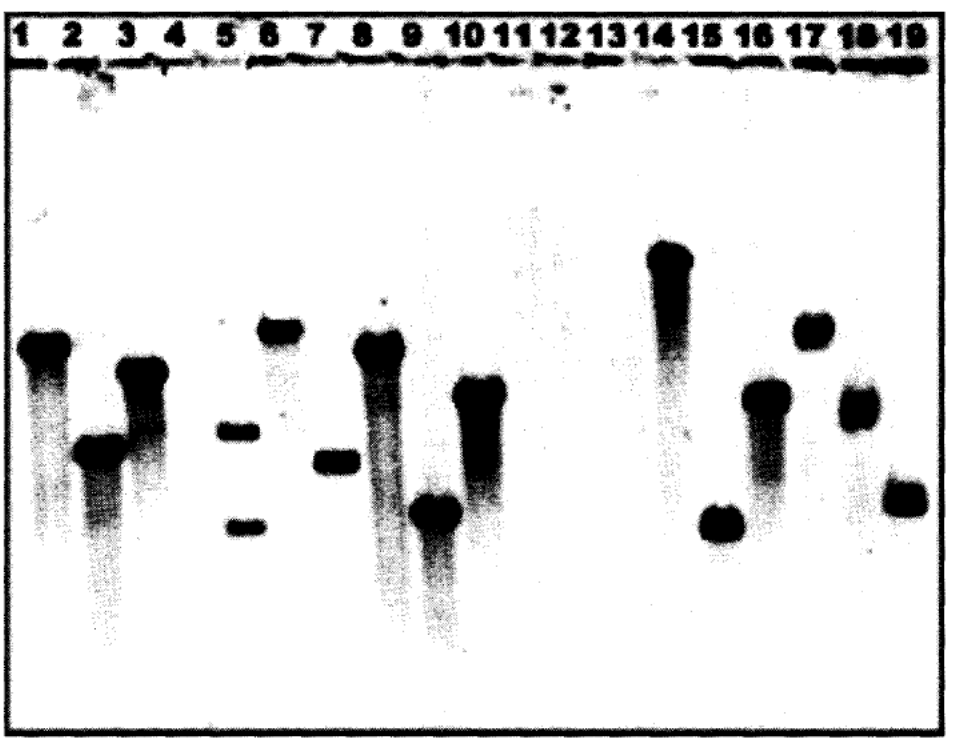
FIGURE 1: The trichodiene synthase gene from *Stachybotrys chartarum*.



The DNA sequence of the coding portion of the gene is in capital letters. Non coding nucleotide sequence is in small letters. The single intron is underlined with a dashed line. Numbering for the nucleotides starts at the start codon. The amino acid sequence is given in as designated by single capital letters below the nucleotide sequence. The letter for each amino acid is aligned below the middle nucleotide of the triplet codon.

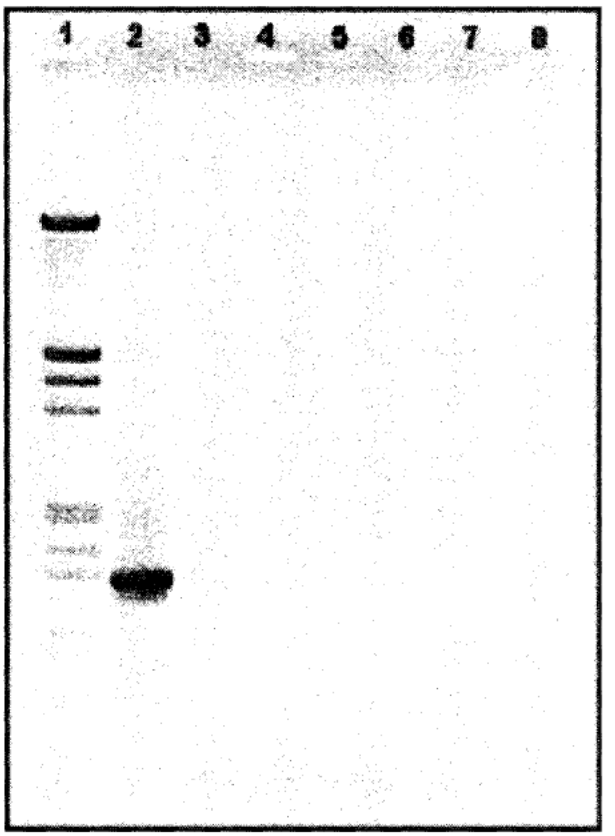
The sequence of the gene for this mycotoxin biosynthesis enzyme may provide a diagnostic tool for detecting the presence of cryptic contamination in homes and workplaces. To test this potential we selected DNA primers from nucleotide sequence regions that show little homology with the corresponding sequences of other fungi. These primers were used in PCR experiments to see if PCR could be used as a diagnostic tool to detect the presence of *S. chartarum* DNA. DNA was isolated from a number of fungi that contain TS and a related fungus that does not contain the gene. The presence or absence of the gene was confirmed by Southern blot analysis, Figure II. PCR was performed on all the fungal DNAs using the *Stachybotrys* primers. Figure III shows that conditions can be developed to specifically detect the *Stachybotrys* trichodiene synthase gene.

FIGURE II: Southern blot analysis for presents of the trichodiene synthase gene.



Lanes 1-3 contain *Stachybotrys chartarum* DNA; lanes 5-7 contain *Fusarium sporotrichioides* DNA; lanes 8-10 contain *Memnoniella* sp. DNA; lanes 11-13 contain *Myrothecium cinctum* DNA; lanes 14-16 contain *Myrothecium roridum* DNA; lanes 17-19 contain *Trichoderma viride* DNA. DNA's in lanes 1, 5, 8, 11, 14, 17 were digested with *Eco*R I; DNA's in lanes 2, 6, 9, 12, 15, 18 were digested with *Hind* lll; DNA's in lanes 3, 7, 10, 13, 16, 19 were digested with *Pst* l. Lane 4 contains Lambda DNA digested with *Hind* lll.

FIGURE III: PCR of various fungal DNA's with *Stachybotrys* specific primers.



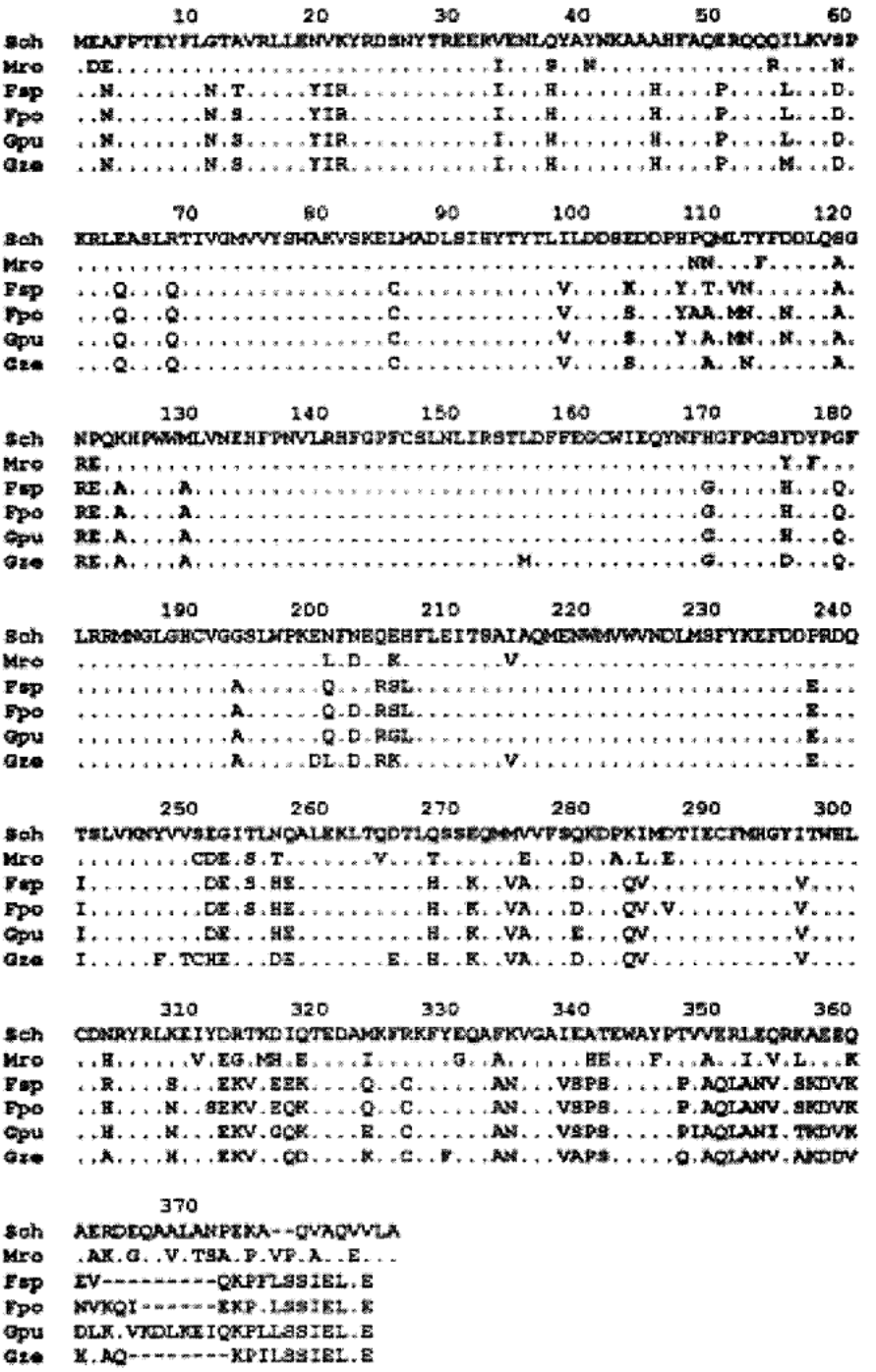
The following lanes contain the results of PCR reactions for the following genomic DNA's: lane 2, *Stachybotrys chartarum*; lane 3, *Fusarium sporotrichioides*; lane 4, *Memnoniella* sp.; lane 5, *Myrothecium cinctum*; lane 6, *Myrothecium roridum*; lane 7 *Trichoderma viride*. Lane 1 contains Lambda DNA that was digested with both *Hin*D lll and *Eco*R I; lane 8 is a control reaction with no DNA added to the PCR reaction. The PCR reaction was for 33 cycles with the following thermal profile: denaturation at 94ªC for 50 sec.; annealing at 65ªC for 50 sec.; extension reaction at 72ªC for 2 ½ min.

DISCUSSION

In this paper, we report the cloning and sequencing of the gene and the cDNA for the first dedicated step in the biosynthetic pathway for trichothecene mycotoxins. A comparison of all of the trichodiene synthase genes that have been sequenced so far indicates that all of them contain a single, small intron (59 to 75 nucleotide pairs) in the identical position in the gene. The intron falls within the codon of the conserved phenylalanine at position 157 of the protein sequence. The intron of *Stachybotrys* with 75 nucloetide pairs is the largest reported so far.

Figure IV aligns the amino acid sequences for all known trichodiene synthases. The comparison shows that the amino acid sequence of trichodiene synthase is quite highly conserved in

FIGURE IV: Amino acid sequence alignment of trichodiene synthase proteins.



Sch stands for *Stachybotrys chartarum*. Mro means *Myrothecium roridum*. Fsp is *Fusarium sporotrichioides*. Fpo is *F. poae*. Gpu is *Gibberella pulicaris*. Gze is *G. zeae*. Dots mean that the amino acid is identical at that position; dashes mean that a gap has been introduced for alignment purposes.

fungi with most of the divergence in amino sequence being clustered in the C-terminus of the protein. The significance of this requires further work but suggests that the active site of the protein is not close to the C-terminus. Knowledge of the sequences of the genes for mycotoxins in different fungi may have a direct application in trying to detect fungal contamination using molecular techniques. We have explored this potential by showing that selected sequences can specifically amplify the *Stachybotrys* gene while showing no reaction to the gene of other fungi. The application of this approach awaits further work, but could be useful in detecting the presence of *Stachybotrys* spores in aerosol samples.

ACKNOWLEDGEMENTS

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MICROSCOPIC FUNGI AND THEIR METABOLITES IN DWELLINGS - A BIOASSAY STUDY

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ABSTRACT

The ciliostatic effect of chloroform-extractable endo- and exocellular metabolites of the most frequently isolated fungal strains from growth in dwellings: *Alternaria sp., Aspergillus glaucus* group, *A. versicolor, Cladosporium sphaerospermum, Penicillium sp., P. chrysogenum, Stachybotrys chartarum, Trichoderma viride* and *Ulocladium sp.* was studied on tracheal cilia from day old chicks in vitro. Biomass extracts from *Alternaria sp.* and *A. versicolor* as well as exocellular extracts from *P. chrysogenum, S. chartarum I, T. viride* and *Ulocladium sp.* stopped cilia movement during the first 24 hours. Biomass extracts of *P. chrysogenum, S. chartarum I* and *T. viride* as well as media extracts from *Alternaria sp., C. sphaerospermum* and *Penicillium sp.* stopped cilia movement between 24 and 48 hours. Between 48 and 72 hours the biomass extract of an *A. glaucus gr.* isolate and media extracts of *A. versicolor* and *S. chartarum II* showed activity while the other extracts (cellular from *C. sphaerospermum, S. chartarum II* and *Ulocladium sp.* and media one from *A. glaucus gr.*) showed no activity. The results are discussed in relationship with health status of people living/working in moldy buildings.

KEY WORDS

Indoor environment, chronic respiratory symptoms, microscopic fungi, fungal chloroform-extractable endo- and exometabolites, ciliated epithelium, tracheal organ cultures, ciliostatic effect.

INTRODUCTION.

There are propagules and toxic metabolites from many different filamentous fungi in the home or working environment. People living/working in moldy buildings are often frustrated because the symptoms they experience are not substantiated in immunochemical analyses. Adults and especially children living in moldy houses exhibit symptoms primarily involving the respiratory tract (Pasanen et al. 1993; Summerbell et al. 1992). The nasal, tracheal and bronchial epithelia contain ciliated cells and mucous production and ciliary clearance provides the first line of defense of the respiratory tract against foreign substances. An *in vitro* model using tracheal rings from day old chicks is an ideal method for the study of the ciliostatic activity of mold metabolites (Jesenská and Bernát 1994; Piecková and Jesenská 1996, 1997).

In this study, we isolated filamentous fungi from growth in dwellings and studied the ciliostatic effects of endo- and exocellular metabolites of the most frequently isolated strains in tracheal organ cultures.

MATERIALS AND METHODS.

Scratch samples were taken from moldy walls in dwellings in Slovakia and Denmark and cultivated on the Sabouraud agar and on Sabouraud agar enriched with NaCl (7.5 %) at 25 and 37[a]C for 10 d. The most frequently isolated strains: *Alternaria* sp., *Aspergillus glaucus* gr., *A. versicolor*, *Cladosporium sphaerospermum*, *Penicillium* sp., *P. chrysogenum*, *Stachybotrys chartarum* (2 strains), *Trichoderma viride* and *Ulocladium* sp. were cultivated as a stationary culture in a 500-mL Erlenmeyer flask with 200 mL of liquid medium: yeast extract (2 %) and sucrose (10 %) at 25[a]C for 10 d. Chloroform extracts of cultivation media and biomass were prepared after their filtration. Day old chick tracheal organ cultures were prepared according to Nair et al. 1970.. Ciliary movement in tracheal rings was observed microscopically during incubation in a minimal essential medium according to Eagle with Earle's salts (Nair et al. 1970) and added crude chloroform extracts (20 μg in 1 ml Me2SO) after 24, 48 and 72 hours.

TABLE I. The effect of chloroform-extractable metabolites from medium (M) and biomass (B) of fungi isolated from moldy walls on the respiratory tract cilia movement of day old chicks *in vitro*

Fungus	**Extract**[a]	**Movement of cilia after days**		
		1	**2**	**3**
Alternaria sp.	M	+	-	-
	B	-	-	-
Aspergillus	M	+	+	+
glaucus group	B	+	+	-
A. versicolor	M	+	+	-
	B	-	-	-
Cladosporium	M	+	-	-
sphaerospermum	B	+	+	+
Penicillium sp.	M	+	-	-
	B	+	+	nd
P. chrysogenum	M	-	-	-
	B	+	-	-
Stachybotrys	M	-	-	-
chartarum I	B	+	-	-
S. chartarum II	M	+	+	-
	B	+	+	+
Trichoderma	M	-	-	-
viride	B	+	-	-
Ulocladium sp.	M	-	-	-
	B	+	+	+
Controls[b]	(a), (b)	+	+	+

[a]M - medium extract (exometabolites), B - biomass extract (endometabolites)
[b]pure cultivation medium (a) and cultivation medium with Me2SO (b)
nd - not determined.

RESULTS

The ciliostatic activities of the fungal exo- and endometabolites are given in the Table I. Biomass extracts from *Alternaria* sp. and *A. versicolor* as well as exocellular extracts from *P. chrysogenum*, *S. chartarum* I, *T. viride* and *Ulocladium* sp. stopped cilia movement during the first 24 hours. Biomass extracts of *P. chrysogenum*, *S. chartarum* I and *T. viride* as well as media extracts from *Alternaria* sp., *C. sphaerospermum* and *Penicillium* sp. stopped cilia movement between 24 and 48 hours. Between 48 and 72 hours the biomass extract of an *A. glaucus* gr. isolate and media extracts of *A. versicolor* and *S. chartarum* II showed activity while the other extracts (cellular from *C. sphaerospermum*, *S. chartarum* II and *Ulocladium* sp. and media one from *A. glaucus* gr.) showed no activity.

DISCUSSION.

It is well known that viable and non-viable fungal propagules in the indoor environment are important allergens for a considerable group of allergic individuals, e. g. *Alternaria* sp. is usually considered the important fungal allergen (Montemuro and Visconti 1992). But there are also non-allergenic health problems without a clear origin in occupants of moldy houses (e. g. Summerbell et al. 1992), and, in this connection, there are only very particular knowledge on role of volatile compounds and other toxic fungal metabolites in affecting of respiratory organs yet (Jesenská and Bernát 1994, Larsen and Frisvad 1995, Piecková and Jesenská 1995).

In this study, fungi were isolated from the walls of moldy dwellings. It was found that some of the investigated strains, especially *Alternaria* sp., *A. versicolor*, *P. chrysogenum*, *S. chartarum*, *T. viride* and *Ulocladium* sp., were able to produce compounds with the strong ciliostatic activity (they stopped the movement of cilia during the first 24 h of the action). It is possible that some known mycotoxins, usually produced by the strains of the tested fungi, e. g. sterigmatocystin - the best known mycotoxin of *A. versicolor*, with the strong ciliostatic activity (Jesenská and Bernát 1994), were present in the crude extracts and were responsible for their ciliostatic effect observed in the experiment.

CONCLUSION.

Inhibited ciliary movement in the airways of individuals who are exposed to mold spores and metabolites during extended periods may be the first stage in the development of chronic respiratory symptoms. More research is needed to understand this complicated problem.

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MOISTURE, MOLD AND HEALTH IN APARTMENT HOMES

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ABSTRACT

The prevalence of observations of moisture or mold, and the respiratory health status of the occupants, was surveyed in a random sample of 120 apartment buildings. The buildings and two apartments from each were given a walk-through inspection and all the signs of moisture and mold recorded using questionnaires and checking lists by civil engineers. A health questionnaire was sent to the occupants. In 60% of the apartments, signs of moisture damage could be observed, and 42% of the apartments were assessed to be in need of repair because of the moisture observations. Respiratory symptoms, such as cough, nocturnal cough and dyspnea, sore throat, hoarseness, rhinitis, nasal bleeding and impaired sense of smell were significantly associated with the observations of moisture, as well as sinusitis (OR=2.58). When the exposure was defined as mold present the symptom findings were almost similar. The results show the health-based importance of good maintenance.

KEY WORDS:

Apartment house, moisture, mold, exposure, health effects

INTRODUCTION

Dampness, condensation or moisture causing wetting of materials may lead to microbial growth. In buildings, this microbial growth may damage buildings and lower the quality of indoor air. The principal factors causing dampness can be leaks in ceilings, walls, windows, roofs or piping. Dampness and mold growth in dwellings have been associated to a number of respiratory health effects (Dales et al. 1991). Since excess moisture in structure is the main factor which permits growth of these microorganisms, observations of dampness and moisture can be used as a proxy of indoor microbial problem and subsequent exposure to microbial emissions (Brunekreef, 1992). The aim of this study was to determine the prevalence of moisture problems in the apartment buildings in Finland and the possible association between moisture and mold observations and respiratory symptoms among a randomly selected population living in apartment buildings.

MATERIAL AND METHODS

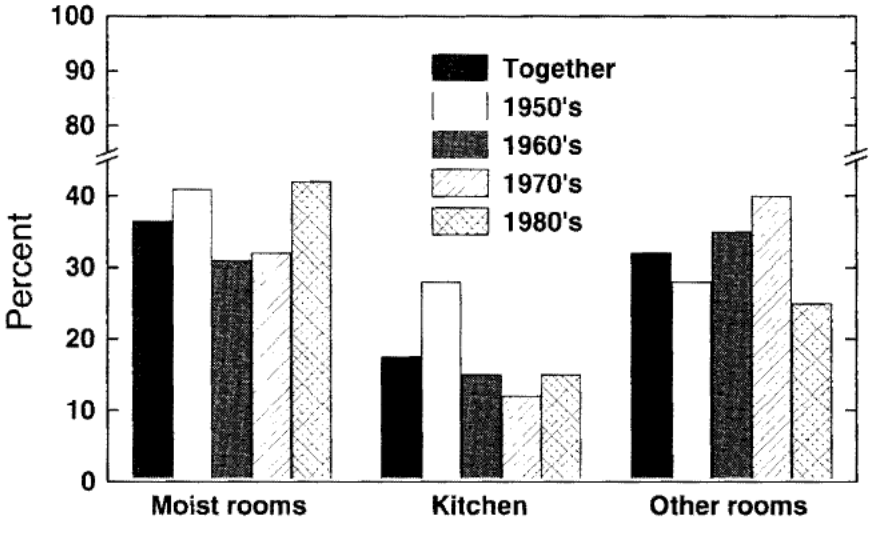
A random sample of 120 apartment buildings, selected randomly from building registries, and two apartments from each building (N=240), were carefully inspected by a civil engineer, specially trained for the standardized procedure (Nevalainen et al. 1998), and all the observations of moisture or moisture repair were recorded. The survey was completed with surface moisture recorders. The homes were classified as being moisture absent if there was no damage or only surface materials were temporarily wetted, and as moisture present if surface finishing was detached or structures had to be dried or replaced.

Health questionnaires were sent to each individual living in these apartments before the civil engineer's visit. In all, 318 adults returned the questionnaire. At least one adult returned a questionnaire in 78% of all the apartments. Exposure was defined in two different ways. Exposure to moisture was defined according to civil engineers observations. Exposure to molds was defined according to occupants' observations on visible mold or odor of mold. Prevalence of respiratory symptoms and infections between exposed and non-exposed groups were compared by multivariate logistic regression. Potential confounders for the health effects (age, gender, allergy and pets) were included in the model. Odds ratios were calculated as well as 95% confidence intervals (CI) for the point estimates of the exposure variables. SAS (Statistical Analysis System) software was used (SAS 6.09 for Alpha).

RESULTS AND DISCUSSION

In 60% of the apartments there was at least one sign of moisture present. This includes all the signs of moisture or a moisture repair. In all, 42% of the apartments were assessed as in need of either a repair or more specific examination while the same figure in the single-family hous-

FIGURE 1: Moisture problems in percents in different facilities in different decades in apartment homes.



es was 55% (Nevalainen et al. 1998). Most moisture problems were in apartments built in the 1950's and in the 1970's and least in the 1960's.

The location of the observations in the different rooms: moist rooms (bathrooms, saunas, laundry etc.), kitchen and other facilities (hall, living room etc.) is shown in Figure I. A total of 22 health outcomes were studied. For 19 health outcomes the proportion of those with the illness was higher in the adults exposed to moisture than respective non-exposed individuals (Table I). Out of these 19 outcomes 9 symptoms were statistically significant. The three health outcomes which did not show an increase were eczema, atopic eczema and allergic eczema.

TABLE I: The proportions of the occupants with respiratory or non-respiratory illnesses and odds ratios for the association between moisture present and moisture absent in the past 12 months.

	Moisture absent N=172	**Moisture present N=143**		
Infection or symptom	n (%)	n (%)	OR	95%CI
Common cold	109 (64)	102 (72)	1.18	0.67-2.06
Tonsillitis	5 (3)	10 (7)	3.00	0.87-10.35
Otitis	6 (4)	7 (5)	0.99	0.30-3.31
Sinusitis	19 (11)	36 (25)**	2.58	1.31-5.06
Bronchitis	17 (10)	18 (13)	1.61	0.74-3.52
Cough without phlegm	24 (15)	39 (28)**	2.00	1.08-3.69
Cough with phlegm	34 (21)	48 (36)*	1.94	1.11-3.40
Nocturnal cough	8 (10	27 (31)**	5.78	2.14-15.61
Nocturnal dyspnea	4 (10)	13 (29)*	9.58	1.61-56.91
Hoarseness	68 (49)	78 (63)*	1.64	0.95-2.83
Sore throat	82 (59)	94 (75)**	1.73	0.97-3.09
Rhinitis	119 (80)	114 (90)*	1.48	0.68-3.22
Nasal bleeding	27 (20)	42 (34)**	2.32	1.25-4.32
Impaired sense of smell	29 (21)	36 (30)	2.06	1.07-3.96
Eczema	36 (22)	22 (16)	0.64	0.33-1.21
Atopic eczema	12 (7)	5 (4)	0.55	0.17-1.76
Allergic eczema	22 (14)	12 (9)	0.52	0.22-1.21
Eye irritation	63 (39)	64 (47)	1.49	0.88-2.51
Nausea	43 (32)	50 (42)	1.36	0.77-2.41
Headache	114 (77)	104 (82)	0.94	0.48-1.83
Fatigue	138 (90)	124 (95)	1.85	0.65-5.23
Difficulties in concentration	55 (41)	58 (48)	1.16	0.68-1.97

* $p < 0.05$, ** $p < 0.01$

Odds Ratios adjusted for smoking, age, gender, allergy, pets indoors and atopic predisposition.

When the exposure was defined as mold, based on the occupants observations, the results were very similar.

CONCLUSIONS

Objective observations of moisture were associated with a number of respiratory health effects. Repair of moisture faults and good maintenance of buildings to control, prevent and detect moisture problems could prevent development of health consequences.

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TOXIGENIC MICROBES IN INDOOR ENVIRONMENT: IDENTIFICATION, STRUCTURE AND BIOLOGICAL EFFECTS OF THE AEROSOLIZING TOXINS

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ABSTRACT

Almost 500 pure cultures, bacteria and fungi, were isolated and identified to genus or species level from indoor environments where the occupants were suffering health problems. Over 80 different taxa were identified. Seven bacterial species classified to Hazard Group 2 were found. Extracts prepared from the building materials and from the pure cultures, were tested for their effects on cellular energy production and conservation using boar spermatozoon and human NK cells as test cells. Strains giving toxic responses were found among strains from 11 taxa. Cell free extracts prepared from cultures of *Bacillus cereus*, *Bacillus licheniformis*, *Streptomyces griseus*, a new species of *Nocardiopsis*, *Stachybotrys chartarum* and *Trichoderma sp* were shown to contain toxins that depleted the spermatozoon of ATP and destroyed plasma membrane integrity. Strains of *Streptomyces griseus* and *Bacillus cereus* were shown to produce toxins that caused mitochondrial swelling and programmed NK-cells towards apoptosis. These toxins were dodecadepsipeptides and operated as K^+ ionophores across the mitochondrial membrane. Strains of *Bacillus licheniformis* and *Trichoderma* produced toxins that depleted the spermatozoon of ATP. The toxins were nonenzymatic, highly hydrophobic, robust molecules of small size ($<10^4$ g mol^{-1}), effective at low concentration (ppb). We propose that the building

related health symptoms of the occupants exposed to one or several of the taxa indicated above, may be caused by microbially emitted toxins. We discuss possible mechanisms of human toxicity.

INTRODUCTION

Building materials with excessive microbial growth have been shown to connect to symptoms of human illness in numerous studies (reviewed by Reijula, 1998; Rylander, 1995, 1998, Johanning, 1995; Hodgson 1995). When no infectious disease (i.e. colonization of humans by the microbes) was observed, the illnesses were thought to relate to allergenic, toxic or immunoreactive responses among those exposed.

We reasoned, that since the typical moisture-problem-house symptoms include eye irritation, fatigue, tiredness and increased frequence of trivial infections (Molhave, 1998), the search for toxins should involve methods suitable for detecting membrane damage, suppression of cellular energy metabolism and / or interference of the immune defense. We found several novel, potentially hazardous agents in moisture problem buildings and animal sheds and describe them in this paper.

MATERIALS AND METHODS

Twenty-six schools and children's day care centers, 6 university offices, one hospital ward and 4 private residences were sampled. The sampling and analyses of airborne and settled dusts and indoor building materials were carried out in 1992-1998 by methods described elsewhere (Peltola et al. this volume, Andersson et al. 1995, 1997, 1999). The dusts, the building materials and the cultured organisms were tested for toxicity as crude water extracts or as methanol (100%) extracts for mitochondrial (Andersson et al. 1997, 1998), cell membrane damaging toxicity (Andersson et al. 1998b, Windsor & White, 1993) using boar spermatozoa as the test cells (Juonala et al. 1998). Viability of lymphocytes was assayed by ^{51}Cr-release assay (Somersalo et al. 1995), the proportion of apoptotic cells by morphology after giemsa staining, and attenuation of the cytotoxicity of natural killer cells towards K562 lymphoma cells was assayed by ^{51}Cr-release as described (Somersalo et al. 1995). Inhibition of growth of *Corynebacterium renale*^{T} was measured according to Crupper et al. 1997.

RESULTS AND DISCUSSION

Microbial taxa found in air, dust and materials in buildings related to ill health. We analyzed the microbial quality of air, settled dusts and building materials from water damaged buildings where the occupants were suffering from building related health problems in Finland. Table I summarizes the more frequent genera and species detected. It shows that several potentially hazardous bacterial species were found: *Acinetobacter calcoaceticus*, *Acinetobacter lwoffii*, *Bacillus cereus*, *Gordonia bronchialis*, *Klebsiella oxytoca*, *Nocardiopsis dassonvillei*, and *Moraxella osloensis*, all classified as Hazard Group 2 of the European Classification (Anonymous, 1995, 1998). Unprotected exposure to these organisms may cause disease in human. Most of the detected

taxa occurred airborne, sometimes at high density (>10^5 cfu m^{-3} or g^{-1} of dust) (Table I). In addition, *Stachybotrys chartarum*, a recognized causative agent of indoor air disease was found in many of the studied 35 moisture damaged buildings.

TABLE I: Microbial species and genera isolated in Finland 1992-1998 from schools, children's day care centers and homes where the occupants suffered health symptoms. The barns and swine confinement buildings were analyzed similarly. Only taxa of which the emergency significantly exceeded that found in local outdoor air, are reported. The table summarizes the taxa identified among the over 400 bacterial pure cultures and 80 fungal isolates. To obtain the pure cultures, indoor samples (air, dusts, building materials) were resuscitated and then cultured at 16ºC, 22ºC and 50ºC. For experimental details see Andersson at al., 1995, Andersson et al. 1997 and Peltola et al. this volume. Viable counts obtained are indicated in the table as follows: +++, >10^6; ++, 10^3..10^6; +, 10...102 cfu g^{-1} or m^{-3}. Hazard Groups indicated are as defined by European legislation (Anon. 1995, 1998). Hazard group is indicated only when the species name is known.

Taxon		Animal sheds		Public & private Buildings		
Genus / species name	**Hazard group**	**Cow Air/dust**	**Swine Air/dust**	**Air / building mat.**	**Settled Dust**	**Reference***
Mesophilic or psychrophilic actinomycete bacteria						
Agromyces sp		+++				B
Arthrobacter ramosus	1			+		B
Arthrobacter pascens	1	+++			++	B
Arthrobacter sp		+++		++	++	A
Brevibacterium sp			+++	+		B
Clavibacter sp		+++		+		B
Corynebacterium sp		+++			+++	B
Curtobacterium sp			++			B
Dietzia sp				Gypsum plate		D
Gordona bronchialis	2			++		C
Microbispora sp		+++				B
Mycobacterium murale sp nov	?			Gypsym plate		G
Nocardiopsis sp nov					+++	H
Nocardiopsis alba	1			Gypsum plate		D
N. dassonvillei	2	+++				D
Rhodococcus, Gordonia sp		+++	+++	+	++	A, B, J
Rhodococcus fascians	1			+	++	C
Spirillospora sp				+		B
Streptomyces sp		+++	+++	+	+++	B
Streptomyces albidoflavus	1			Gypsum plate		D
Streptomyces griseus	1	+++	+++	+	+++	D
Williamsia muralis gen nov sp nov	?			Gypsum plate		E

TABLE I cont.

Taxon		Animal sheds		Public & private Buildings		
Genus / species name	Hazard group	Cow Air/dust	Swine Air/dust	Air / building	Settled Dust mat.	Reference*
Other mesophilic or psychrophilic gram positive bacteria						
Bacillus alvei	1	+ + +				B
B. cereus	2	+ + +	+ +		+ +	B
B. licheniformis	1	+ + +		+	+ +	B
B. mycoides	1	+ + +				B
B. psychrophilus	1			+ +		B
B. subtilis	1		+ + +		+ + +	B
Bacillus sp					+ + +	B
Brevibacillus brevis	1		+ + +	+	+ +	B
Deinococcus sp				+	+ +	B
Micrococcus luteus	1	+ + +	+ + +	+	+ + +	C
Paenibacillus pabuli	1	+ + +			+ + +	B
Paenibacillus gordonae				+ +		C
Staphylococcus sp			+ + +	+	+ + +	B
Mesophilic or psychrotrophic gram negative bacteria						
Acinetobacter calcoaceticus	2			+	+ +	B
Acinetobacter lwoffii	2			+		B
Acinetobacter sp		+ + +	+ +			B
Agrobacterium sp		+ + +				A
Brevundimonas sp		+ + +				B
Chryseomonas sp			+ + +		+ +	B
Cytophaga sp		+ + +				B
Enterobacter sp				+		B
Flavobacterium sp			+ + +			B
Klebsiella oxytoca	2			+		B
Microbulbifer sp			+ + +			B
Moraxella osloensis	2		+ + +	+		C
Pantoea sp		+ + +			+ +	B
α-proteobacterial new genus		+ + +				B
Pseudomonas chloroaphis	1	+ + +				B
Ps cichori	1		+ + +			B
Ps stutzeri	1	+ + +				B
Ps syringae	1	+ + +				B
Ps marginalis	1		+ + +			B
Ps putida	1		+ + +			B
Sphingomonas sps		+ +	+ +			B
Xanthomonas sp		+ + +				B

TABLE I cont.

Taxon		Animal sheds		Public & private Buildings		
Genus / species name	Hazard group	Cow Air/dust	Swine Air/dust	Air / building	Settled Dust mat.	Reference*
Thermotrophic bacteria						
Bacillus sp			+ +	+		A, B
B. amyloliquefaciens	1				+	A
B. licheniformis	1	+ +	+ +			B
B. subtilis	1	+ +	+ +			B
B. pumilus	1	+ +				A,B
Brevibacillus brevis	1				+	B
Paenibacillus macerans	1	+ +				A
Sacchraromonospora sp			+ +			B
Thermoactinomyces sacchari	1	+ +	+ +			B
Thermoactinomyces vulgaris	1	+ +	+ +		+ +	B
Thermomonospora sp		+ +				B

Fungi	Dust	Building material	Reference
Aspergillus ochraeus Wilhelm	+ +		F, J
Aspergillus versicolor Vuill. Tiraboschi		Mineral wool	J
Chaetomium globosum Kunze		Mineral wool	J
Cladosporium herbarum (Pers,:Fr.) Link	+ +	Concrete, mineral wool	J
Cladosporium sphaerospermum Penz.		Concrete	J
Mucor hiemalis Wehmer		Gypsum liner	J
Mucor plumbeus Bonord		Gypsum liner	J
Paecilomyces aurantiogriseum Dierckx		All samples	J
Paecilomyces variotii Bain		Mineral wool	J
Penicillium citreonigrum Dierckx		Wall board	A, J
Penicillium citrinum Thom		All samples	J
Penicillium sp (7 taxa)	+ +	Board, concrete, gypsum	F, J
Stachybotrys chartarum (Ehrenb.) Hughes		Gypsum liner	A, F, J
Trichoderma sp		Wood	C, J
Tritirachium oryzae (Vincens) de Hoog	+ +		J
Ulocladium sp	+ +	Concrete	J

*

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A. Andersson et al, 1997; **B.** Andersson et al., 1999 ; **C.** Andersson, M. A., Mikkola, R., Peltola, J., & Salkinoja-Salonen, M.S., 1998, Unpublished. ; **D.** Andersson et al., 1998b; **E.** Kämpfer et al., 1999; **F.** Peltola et al., this volume; G. Vuorio et al., 1999; **H.** Andersson, M.A, Kroppenstedt, R.M., Salkinoja-Salonen, M.S. & Rainey, F.A., unpublished; **I.** Andersson et al., 1998a; **J.** Latva-Kala et al, ,1998.

TOXIN PRODUCTION BY MICROBES ISOLATED FROM MICROBIALLY AFFECTED BUILDINGS.

Table II lists the tests used to track toxins produced by the indoor microbes. Among the taxa listed in Table I, nine different types of toxic responses were found. Many isolates produced

TABLE II. Biological methods used to detect toxicity and cell damage in sick building related microorganisms.

Test cell	Parameter measured	Toxic response	Examples of causative microbial Species found in Indoor environment	Reference
Boar sperm cell	Motility	Cessation of movement	Several *Bacillus* species, *Streptomyces griseus, Nocardiopsis sp, Stachybotrys chartarum, Trichoderma sp*	A, B, C D, G, H
Boar sperm cell	Signal trafficking	Accelerated motility	*Paenibacillus sp*, new genus of ß-proteobacteria	C
Boar sperm cell	Vitality staining	Collapse of cytoplasmic membrane potential	*B. licheniformis, Stachybotrys sp Trichoderma*	H,I
Boar sperm cell	Cellular ATP	Depletion	*B. licheniformis*	C, I
Boar sperm cell	Mitochondrial integrity	Swelling	*B. cereus, Str. griseus*	B, F
Boar sperm cell	Acrosome response	Activation	*B. licheniformis*	C
Human NK cell*	Reactivity towards Leukemia cells	Inhibition of killing activity	*Str. griseus, B. cereus*	F, G
Human NK cell	Signal trafficking	Apoptotic morphology	*B. cereus, Str. griseus*	F, G
Human NK cell	Mitochondrial integrity	Swelling	*B. cereus, Str. griseus, Stachybotrys sp*	F, G, J
Corynebacterium renale ATCC19412	Growth on agar medium	Inhibition of growth / (clearing zone)	*B. licheniformis*	C,I

*NK, natural killer cell

References (as listed):

A. Andersson et al. 1999; B. Andersson et al. 1998a; C. Andersson, M. A., Mikkola, R., Peltola, J., Rainey, F. & Salkinoja-Salonen, M.S., 1998, Unpublished. D. Andersson et al. 1998b; E, Mikkola et al. 1999; F. Paananen et al.1999; G. Timonen, T., Mikkola, R., Paananen, A., Andersson, M.A. & Salkinoja-Salonen, M.S., 1998, unpublished; H. Peltola et al. unpublished; I. Vuorio et al. 1998; Peltola et al. this volume

toxins that paralyzed spermatozoan motility and extremely low concentration (< 1 ng ml^{-1}) and caused mitochondrial swelling in target cells (boar spermatozoa, human peripheral blood lymphocytes) at concentrations (≥100 ng ml^{-1}).

Ionophoric toxins from microbes occurring in indoor materials.

Toxins produced isolates of *Streptomyces griseus* (indoor air) and *Bacillus cereus* (wood) were analyzed. Figure 1 shows an example of the toxin of *Str. griseus* strain 8/ppi, coeluting from RP-HPLC with the model compound valinomycin (Sigma). It was shown by electrospray tandem

FIGURE 1: Purification of sperm motility paralyzing toxin from *Streptomyces griseus* strain 8/ppi, isolated from settled dust at a children's day care center. Cell free extract of the culture had an EC50 of 50 ng dry substance ml^{-1} of extended boar semen determined as the end point of dilution giving ≥ 50% loss of motile sperm cells, equivalent to 10^6 cfu ml^{-1}. The sperm toxic agent was purified from the extract using methanol as solvent, Sep-Pak and Smart HPLC as described elsewhere (Andersson et al. 1998b). The figure shows the HPLC tracings of semipurified toxin and commercial valinomycin.

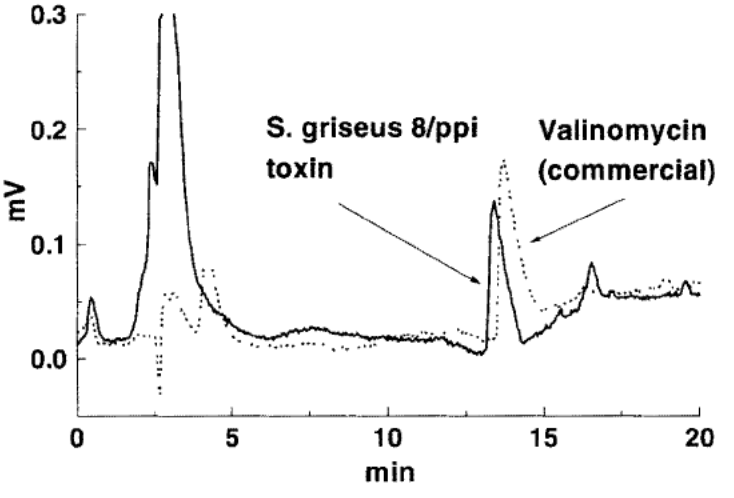
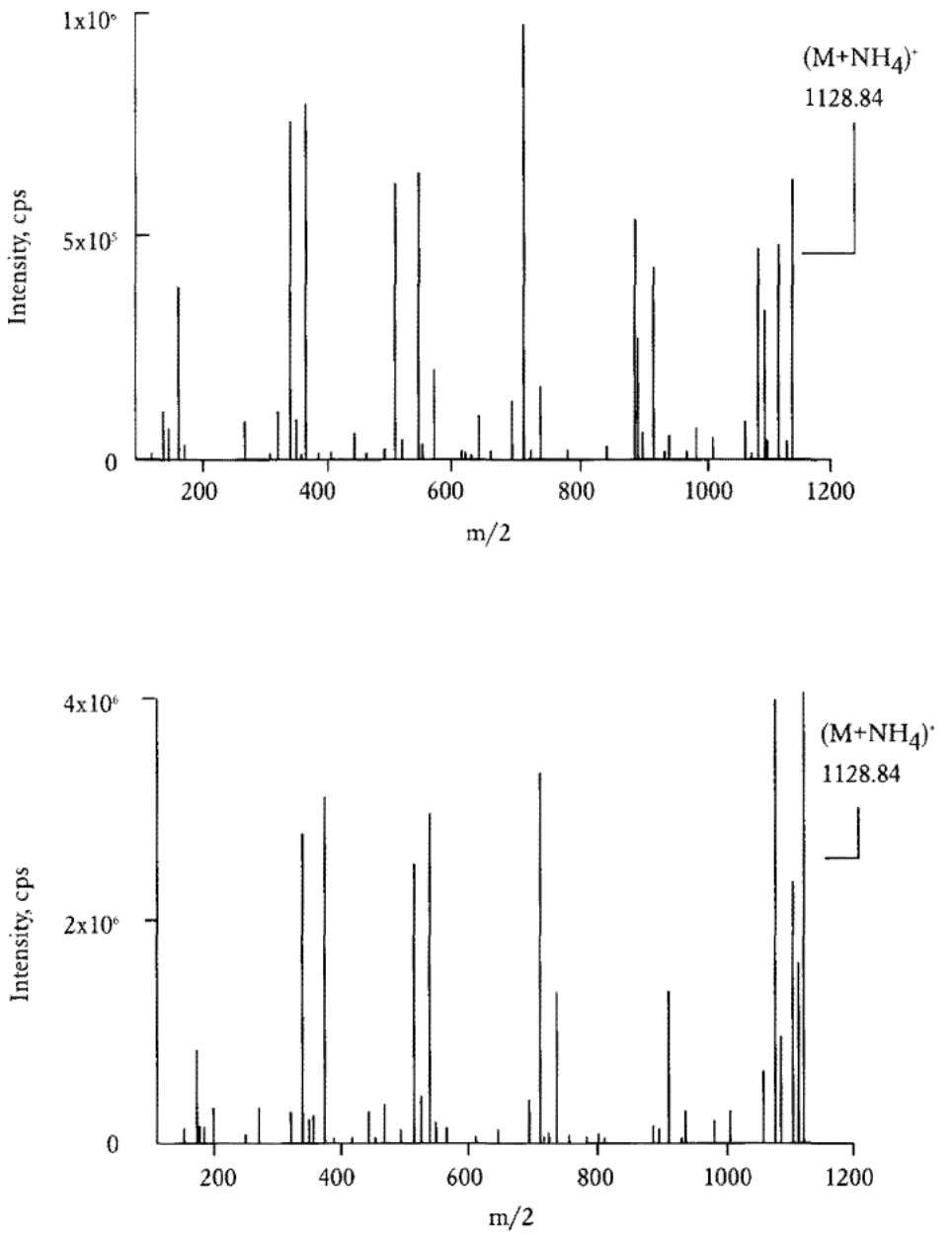


FIGURE II: Electrospray MS/MS spectra obtained for the toxin purified from *S. griseus* strains 8/ppi (top) and commercial valinomycin (bottom). The molecular mass of the ammonium adduct molecular ions are shown. Ammonium acetate (5 mM) was added to the 50% methanolic solution of the toxin before analysis, explaining for the ammonium ion cleavage.



mass spectrometry that the *Str. griseus* toxin was identical to valinomycin, (-Val-OAla-Val-OVal-)3, where OVal and OAla symbolize analogs of valine or alanine, respectively, where -NH2 group of the amino acid was replaced by -OH (Andersson et al. 1998a, 1998b).

The toxins from *Str. griseus* and *B. cereus*, were highly selective K^+ carriers, 3 orders of magnitude over Na^+, H^+ or Ca^{2+} (Mikkola et al. 1999). The *B. cereus* toxin was found identical to the emetic toxin of *B. cereus*, called cereulide (Andersson et al. 1998a). Figure III shows the Maldi-TOF mass spectra of the K^+, Na^+ and NH_4^+ adducts of the *B. cereus* toxin. Molecular modeling proved the perfect match of K^+ ions in the center channel of the circular molecules of valinomycin and the cereulide, -(Oleu-Ala-Oval-Val)3, (Figure IV).

FIGURE III: MALDI-TOF-mass spectrum of purified toxin (cereulide) from the strain *B. cereus* 4810/72. The toxin was purified from the cell free extract as described (Andersson et al. 1998a). The spectrum shows the cereulide molecular ion (K^+, Na^+ and NH_4^+ cationized species).

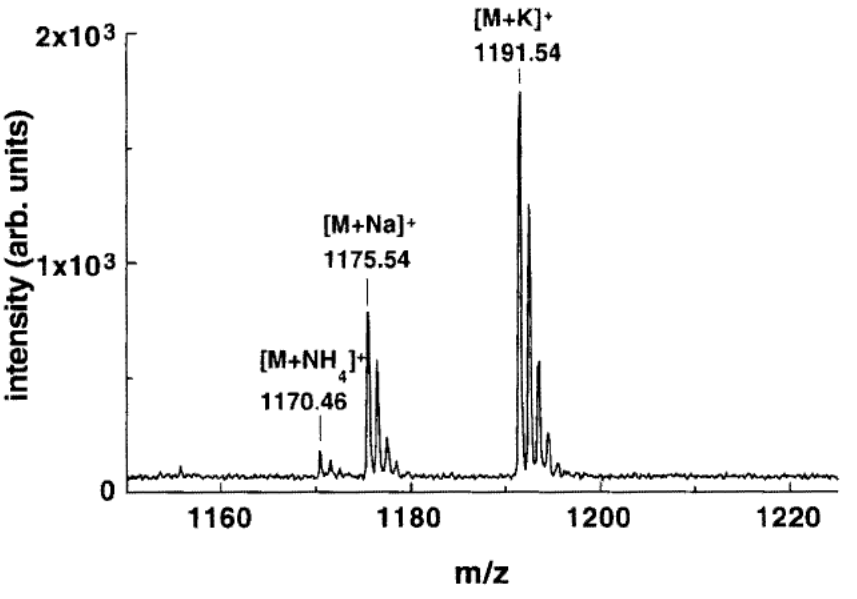
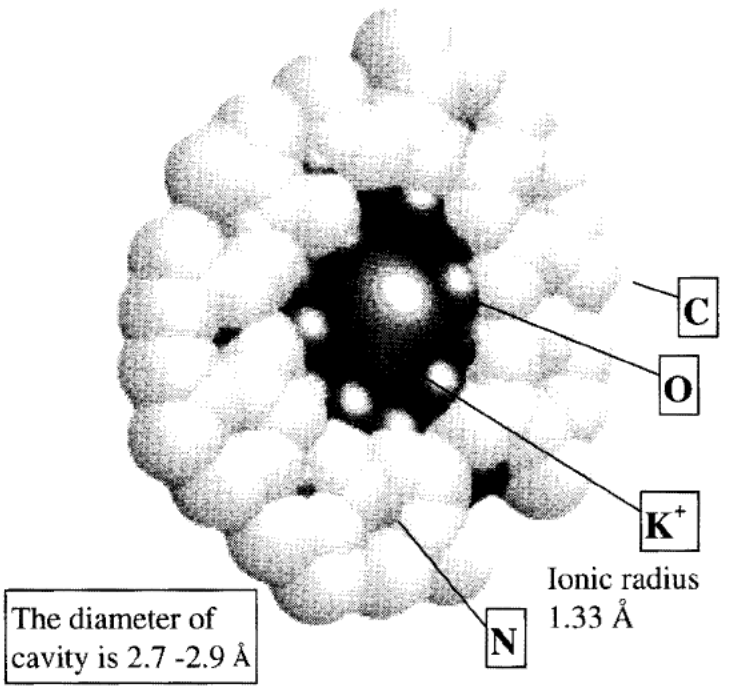


FIGURE IV: Molecular model of valinomycin, the toxin produced by several indoor strains *Streptomyces griseus*, isolated from dust and from indoor building materials of moisture problem buildings. The model shows the perfect match of the ion radius of K^+ into the channel formed in the ring-formed depsipeptide molecule, (-Val-OAla-Val-Oval-)3. The atomic diameter data were taken from Yu. Ovchinnikov (1974). Membrane active complexones. Chemistry and functions. FEBS Letters 44:1-21.

(-Val-OAla-Val-Oval-)3



Color Plate can be viewed on page 672.

Valinomycin is a well known potassium ionophore, produced by *Streptomyces levoris*, *Str. tsusimaensis* (Perkins et al. 1990), and commercially by *Str. fulvissimus* (Merck Index). *Str. fulvissimus* is only distantly related to *Str. griseus* (Andersson et al. 1998b) and not found among the indoor samples. Valinomycin is used commercially as an insecticidal-nematocidal agent (cited in Perkins et al. 1990).

Immune toxicity of indoor bacteria.

Microscopic observation of human NK cells exposed to purified toxins of toxic strains of *B. cereus* and of *Str. griseus* revealed shrinkage of cells and formation of pycnotic nucleus indicating onset of apoptosis. Valinomycin (commercial) was also tested and was found to induce morphological changes in NK cells similar to those caused by the *Str. griseus* and *B. cereus* toxins. Valinomycin is known to induce apoptosis in hepatoma cells via activity increase of cas-

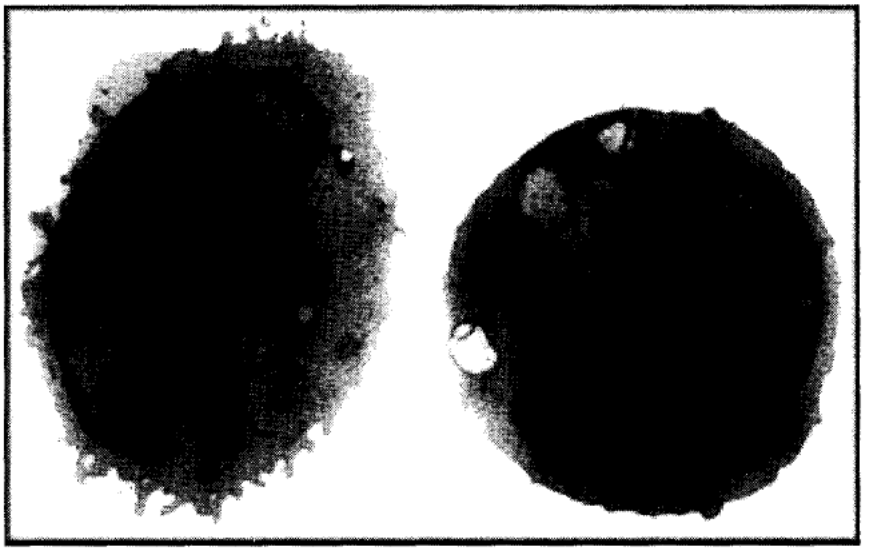
pase-3 like protease but not caspase-1 like protease (Sun et al. 1994), not involving the release of reactive oxygen species from mitochondria nor an increase in intracellular calcium concentration (Inai et al. 1997). Valinomycin induced apoptosis in murine hematopoietic cells by triggering rapid loss of mitochondrial membrane potential (Furlong et al. 1998). Our results indicate that the toxins of *Str. griseus* and *B. cereus* may induce apoptosis in human NK by a mechanism resembling that by valinomycin in ascites hepatoma cells and murine hematopoietic cells.

The purified toxins of *B. cereus* and *Str. griseus* instantaneously inhibited the leukemia-cell killing activity of the NK-cells (Table III). Within 1 min after dispensing the toxin on the lymphocytes, a dramatic inhibition of NK-cell activity was observed. Upon prolonged exposure, these toxins swelled the mitochondria in exposed human peripheral blood lymphocytes (Figure V.) similarly to what was observed for boar spermatozoan (Andersson et al. 1998a, b). Our results strongly indicate that the toxins produced by *B. cereus* and *Str. griseus* may thus be immunotoxic to man. Both toxins are extremely hydrophobic (log K_{ow} of 6.0 and 7.5, respectively) (Mikkola et al. 1999) and are likely to be resorbed into the blood stream as soon as inhaled.

TABLE III: Attenuation of the cytotoxicity of human NK-cells against K562 chronic myelogenous leukemic cells by toxins from *Streptomyces griseus* strain 10/ppi, *Bacillus cereus* strain F5881 and strain F4810/72 and valinomycin.

	Observed cytotoxicity, % after exposure of		
Toxin	**1 min**	**10 min**	**3 h**
Control (reagents only)	52.3	67.2	54.0
Valinomycin (Sigma), 30 ng m^{-1}	23.0	32.6	31.9
100 ng ml^{-1}	ND	ND	35.4
Purified toxins from:			
S. griseus 10/ppi, 60 ng ml^{-1}	28.1	25.9	29.8
B. cereus F5881, 30 ng ml^{-1}	25.1	28.6	32.6
B. cereus F4810/72, 20 ng ml^{-1}	25.2	21.9	30.5

FIGURE V: Transmission electron micrographs of human NK cells. Left panel (A), cell exposed to reagents only; right panel, cell exposed 3 h at 37°C to valinomycin (72 ng ml^{-1}). It is seen that the exposed cells have swollen mitochondria. Bar: 0.5mm.



TOXINS DAMAGING CELLULAR ENERGY GENERATION.

We found indoor taxa of bacteria and fungi emitting toxins that zeroed the cellular concentration of ATP (Table II): *Bacillus licheniformis* (Vuorio et al. 1998). Such toxins may affect the vital organs of our body, such as kidney, heart and brain, requiring steady ATP synthesis to be able to operate. These toxins may be the explanation for the intense fatigue complained by the occupants of many of the buildings where these microbial strains were isolated from (Peltola et al. this volume).

TOXINS AFFECTING SIGNAL TRAFFICKING.

Exposure to extracts of the building materials and certain microbes cultivated from these, induced in spermatozoa the capacitation reaction, for example *Paenibacillus sp* (Table II). The swimming of the spermatozoon became intense, disoriented and disregulated, leading to exhaustion of the motive force. If active on other cells, such toxins may impair the proper operation of motile cells in our body, for example cilia on mucous membranes, lymphocytes, macrophages.

IMMUNOREACTIVE OR SENSITIZING MICROBIAL COMPONENTS.

In dust and wall materials from water damaged children's day care centers we found large amounts of actinomycetes (Table I, see also Andersson et al. this volume), *Mycobacterium murale* (Vuorio et al. 1999), species of *Rhodococcus, Gordonia, Dietzia*, and *Nocardiopsis* (Andersson et al.1999). Highly immunoreactive lipoarabinomannans (LAMs, reviewed by Daffe & Draper, 1998) are exposed on the outer cell membrane (wall) of mycobacteria. The mycobacterial cell

wall also carries an immunodominant protein, and a similar protein occurs on the cell wall of *Streptomyces griseus* (Triccas et al. 1998).

Environmental mycobacteria cause human diseases with an increasing frequency (Guay, 1996). Long-term exposure to mycobacterial cell wall antigens is suspected to provoke autoimmune symptoms in man (discussed by Andersson et al. this volume). Mycobacteria like many other actinomycetes are desiccation resistant and are likely to survive long dry periods of time in buildings (Hirai, 1991). Old water damages, long dry, may emit actinomycetal cell wall fragments smaller than cells and therefore penetrating more efficiently into the alveoli. Considering the above, it is possible that inhaled actinomycetal cell components, even when belonging to nonviable cells or cell fragments, may contribute to the impairment of human health.

MEMBRANE DAMAGING TOXINS.

Many of the bacterial genera and species, listed in Table I, are known as potential emitters of antimycotic, amoebicidic, or surfactant type of molecules. For example, an aminopolyol antibiotic zwittermicin is produced by *Bacillus cereus* (He et al. 1994; Raffel et al. 1996), bacillomycin, surfactin and iturin A by *B. subtilis* (Hourdou et al. 1989, Sandrin et al. 1990), fungicin M, amoebicins and lichenysin A from *B. licheniformis* (Lebbadi et al. 1994; Galvez et al. 1993, Yakimov et al. 1995), coagulin by *B. coagulans* (Hyronimus et al. 1998), antifungal peptides of *B. pumilus* (Munimbazi & Bullerman, 1998), staphylococcin BacR1 of *Staphylococcus aureus* (Crupper et al. 1997), pristinamycin of *Streptomyces* sp (Thibaut et al. 1997).

In a school building as well in animal sheds (Table I), we measured high amounts of *Bacillus licheniformis* and *B. subtilis* (Table I). Biosurfactant type of toxins were isolated from two strains of *B. licheniformis* (Mikkola et al. unpublished). The protective liquid lipid film normally lubricating the eye (cornea) may be damaged by microbially emitted, aerosolized surfactants. *B. subtilis* and *B. licheniformis* have been reported as the major suspect in connection to building related illness also in the US (Thorne, 1993). The emitted biosurfactants may also promote aerosolization and thus potentiate the biological reactivity of lipid-rich bacteria like *Mycobacteria* and other actinomycetes by acting as dispersant.

CONCLUSIONS.

A great variety of toxin emitting bacteria and fungi were observed in indoor air. Toxins with powerful ionophoretic activity, specific for K^+ ions, were isolated from *B. cereus* and *Streptomyces griseus* and were shown to be immunotoxic *in vitro*. Exposure to such toxins may explain the prevalence of reoccurring trivial infections among persons exposed to microbially damaged indoor environment. Being K^+ ionphores, these toxins may also be suspected to exhibit neuronal toxicity. The observed co-occurrence of immunoreactive actinomycetes (*Mycobacterium*) may potentiate immunologic disturbance. Damage to cellular energy generation (ATP) by toxins emitted by indoor bacteria (*Bacillus licheniformis*) may explain the extreme fatigue frequently complained by the affected persons. Membrane damaging toxins were found in toxic strains of *B. licheniformis* and of *Stachybotrys chartarum*, possibly explaining the irritation of eyes and airways. Toxins affecting cellular signal trafficking were detected by their ability to inducer capacitation and acrosome reaction in spermatozoa. Such toxins may relate to the occurrence of

neurologic or psychological disturbance frequently observed with the occupants of sick buildings. Ionophoretic toxins (*B. cereus, Str. griseus*) may potentiate such effects.

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AN ASSESSMENT OF THE OCCURRENCE OF MOULD BIOAEROSOLS IN THE IN- AND OUTDOOR ENVIRONMENT OF SHACKS IN DURBAN, SOUTH AFRICA

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ABSTRACT

The occurrence of fungal aerosols in the shacks of Malukazi squatter settlement (Durban, South Africa) was investigated. An initial questionnaire survey was conducted in 15 homes (shacks) and 10 reference homes followed by detailed measurements of indoor and outdoor bioaerosols using an Anderson sampler. Surface moulds, whenever visible, were sampled and identified. Temperature and relative humidity were also monitored. About 40% (19 out 49) of children in the study homes compared to 20% (reference) reported frequent respiratory tract (RT) symptoms. Most of the shacks lacked proper ventilation and showed signs of dampness and mouldiness. Indoor fungal contaminant levels in control homes were lower than or comparable to those outdoor, whereas 9 of the 15 (60%) shacks showed higher indoor mould levels. It was in these homes that most RT symptoms were reported. Predominant airborne fungal organisms identified included *Aspergillus* and *Penicillium.*

KEY WORDS:

shacks, moulds, bioaerosols, respiratory symptoms

INTRODUCTION

High respiratory disease morbidity has been noted among pediatric outpatients at King Edward VIII Hospital in Durban, South Africa. The majority of these came from the surrounding shack settlements. These settlements are unique in that cheap building materials are frequently used in the construction of the shacks. In a humid and warm environment such as the Durban climate, proper building design is necessary to minimize internal mould growth.

It is now recognized that the airborne environment, both vast and dynamic, is an important medium for the dispersal of a number of agents of infection and contamination, not the least of which are fungal propagules. Furthermore, studies have shown that chronic exposure to toxins produced by indoor moulds, even in small doses, may lead to severe health effects over a long period (Chang-Yeung, 1995; Bjurman & Kristensson, 1992). These toxins constitute an ever-increasing threat to the health status of humans as they may invade and attack almost every organ system in the human body. Inhalation is believed to have a more systemic effect, since it permits the entry and absorption of dissolved substances into the lungs (Seltzer, 1994) and directly into the blood supply.

Biological contaminants do occur in varying proportions in every indoor environment while the potential to produce human illness is ever-present and found to be most prevalent amongst children (Seltzer, 1994). Their rapid development and unique physiological processes make children especially vulnerable to toxins and other pollutants (Lubin & Lewis, 1995). Several studies have confirmed a positive correlation between moldy homes and asthma morbidity (Bjurman & Kristensson, 1992; Mishra et al. 1992).

The purpose of this study was to assess the occurrence of moulds in the shacks of Durban forming part of an initial survey aimed at investigating the correlation between mould bioaerosols and health in these communities.

METHODS

QUESTIONNAIRE SURVEY

Homes were selected according to the building materials and the type of construction method used. Shacks (study homes) were constructed from untreated wood, aluminum sheet, cardboard and plastic with no planning or thought to design while the reference homes were constructed in a more formal manner from brick, glass, wood and aluminum. The general appearance of each home was assessed according to structural damage observed and the degree of dampness, darkness and general disrepair.

A questionnaire with reference to the medical history of each child in the household and the general appearance of the home was administered to each participating home. Included in the questionnaire were queries related to smoking habits of residents and respiratory health and allergies. This questionnaire provided information on the type of respiratory diseases experienced by the residents, the treatment received, frequency of occurrence of these diseases, a summary history of known allergies and information on allergic symptoms experienced by each child.

MOULD SAMPLING

Air and surface sampling was performed in the winter of 1997 under normal operating conditions of each home. A sample of the indoor and outdoor air was taken using the Anderson Single Stage sampler which deposited conidia onto malt extract agar (MEA) plates. The temperature and relative humidity levels in the internal and external environment were also recorded during the sampling period. These plates were incubated (250C) for 5-7 days. Viable colonies were then counted and individual cultures were isolated for further identification purposes.

FUNGAL IDENTIFICATION

Isolated cultures were three-point inoculated onto MEA, yeast extract and Czapek yeast autolysate agars, respectively, and incubated for 5 days (25^{a}C, 30^{a}C). The diameters and visual characteristics of the colonies were used for identification. In addition, light microscopy was employed to observe the fruiting bodies and hyphae of the fungal isolates. Wet mount preparations of the fungal cultures were made using lactophenol cotton blue and dilute toluidene blue to observe the fruiting bodies, hyphal fragments, and conidia shape and size.

RESULTS

CHILD HEALTH QUESTIONNAIRE

In this study we invited mothers to participate in the child health questionnaire survey based on our past experiences on this type of work. Mothers tend to be more co-operative and understand child health issues more because they are directly involved in monitoring their children. Although we administered questionnaires to adults many did not complete them.

The demography of the participants from the study and reference areas summarized in Table I is similar, although sample sizes were different. The distribution of adults and children was similar in both areas. In the study community adults in 60% of the homes reported to be regular smokers compared to 40% in the reference community. The number of homes with visible dampness (46.7%) and mouldiness (40%) in the study area was higher than that in the reference area (30 and 20%, respectively). Also, 55% of homes in the study area reported children with respiratory symptoms versus 30% of those in the reference area.

TABLE I: Summary table indicating demography, smoking status, and respiratory health history of some of the participants in the questionnaire survey.

	Study Area (%)	**Reference area (%)**
Number of participants	91	38
Adults	42 (46,1)	18 (47,4)
Children	49 (53,9)	20 (52,6)
Number of homes with smoking adults		
Smoking	9 (60)	4 (40)
Non-smoking	6 (40)	6 (60)
Number of homes with visible dampness and mouldiness		
Dampness/water damage	7 (46,7)	3 (30)
Mouldiness	6 (40)	2 (20)
Number of homes reporting child respiratory symptoms	8 (53)	3 (30)
Respiratory symptoms of children		
Asthma	5 (10)	2 (10)
Allergic rhinitis	14 (28,8)	2 (10)
No respiratory symptoms	30 (61,2)	16 (80)

In general, 61.2% of children in the study area had neither allergic rhinitis nor asthma compared to 80% in the reference area. Of those reporting symptoms about 38% were in the study area compared to 20% in the reference area.

IN- AND OUTDOOR CONDITIONS

Table II shows the data on mould bioaerosol, temperature, and relative humidity levels in the in- and outdoor environments of Malukazi. Total colony forming units per m^3 of air sampled (CFU/m^3) levels within the external environment of the study area was 147 (range 21 - 307)

compared to 136 (53 - 169) in the reference area. Indoor CFU/m³ levels were generally higher in the study homes; mean levels of 172 (kitchen - range 2 - 358), 106 (living room - range 2 - 260) versus mean levels of 121 (64 - 165) and 75 (2 - 149) in the reference homes, respectively. Although the mean CFUs determined were not significantly different, indoor fungal contaminant levels in reference homes were lower than or comparable to those outdoor. At least 9 of the 15 shacks (60%) showed higher indoor mould than outdoor levels with the kitchen recording the highest levels (maximum recorded 358 CFU/m³). It was most of these homes that showed visible dampness and mouldiness.

TABLE II: Summary statistics for mould bioaerosol, temperature and relative humidity levels in the study and reference areas.

GROUP		N	Mean	SD	Range
CFU - Kitchen*	study homes	15	172	110	2 - 358
	reference homes	10	121	34	64 - 165
CFU - Living room*	study homes	15	106	96	2 - 260
	reference homes	10	75	48	2 - 149
CFU - Outdoor*	study homes	15	147	115	21 - 307
	reference homes	10	136	35	53 - 169
Indoor temp (ªC)	study homes	15	24,6	2,4	21 - 30
	reference homes	10	22,4	0,8	21 - 24
Outdoor temp (ªC)	study homes	15	22,5	3,5	20 - 24
	reference homes	10	21,6	0,8	20 - 24
Indoor relative	study homes	15	56,7	10,8	41 - 74[1]
humidity	reference homes	10	51,0	9,6	36 - 63[1]
Outdoor relative	study homes	15	52,0	11,8	39 - 64
humidity	reference homes	10	49,9	8,8	38 - 61

* CFU - colony forming units per m³ of air sampled were determined using an Anderson sampler and malt extract agar

[1] mean values are significantly different ($p<0,05$)

The ranges of indoor temperature and relative humidity determined were higher in the study homes (21-30ªC, 41-74 %RH) compared to the reference homes (21-24, 36-63 %RH). In fact, an independent sample t-test showed that indoor relative humidity levels in the study homes were significantly different to those in the reference homes ($p=0,03$).

Visible mould was found in 40% of study homes with at least 20% of this number experiencing severe mould growth. Only 20% of the reference homes displayed moderately severe surface mould growth, while the rest of the homes (80%) had no mould growth at all. This compared with 60% of the study homes that lacked visible mould growth. Evaluation of the mycological samples revealed the presence of 22 fungal isolates with the predominance of *Aspergillus* (41%) and *Penicillium* (27%). Other genera which occurred to a lesser degree were the *Acremonium*, *Cladosporium*, and *Eurotium*. Of the Aspergilli and Penicillia present, light microscopy showed that *A. clavatus*, *A. flavus* and *P. glabrum* were the most common.

TABLE III: The number of homes with visible surface mould growth and the extent* thereof.

Score	Study homes (%)	Reference homes (%)
0	9	8
1	1	0
2	2	0
3	0	2
4	3	0

* these were ranked on the scale 0 - 4; where 0 indicates lack of visible mouldiness per $1m^2$, and 4 - severe surface mould growth per $1m^2$

DISCUSSION AND CONCLUSION

This study has established the occurrence of mould bioaerosols in the shack settlement of Malukazi located along the kwaZulu-Natal South Coast of South Africa. The homes are typical of an informal settlement, as they are small dwellings constructed from mainly wood, iron, tin, and cardboard or plastic. Furthermore, the climate of the region is such that high temperature, relative humidity and rainfall levels persist for many months of the year and are amongst the highest in the country. The population density of the region is also considerable, second only to the Gauteng region (Central Statistical Services, 1995). The area is not provided with a refuse removal service thus, necessitating the dumping of refuse in open dump sites proximate to the homes. In addition, taps are communal, as are sanitation facilities and average one per 10 to 15 households.

MOULD BIOAEROSOLS

It is well known that dampness and humidity are among the critical factors for surface mould growth within buildings. According to Flannigan (1994) up to 1150 CFUs of mould is acceptable if the species present reflect the flora outdoors. There were no great differences between the in- and outdoor mycoflora isolated from the households in this study. However, the occurrence of Aspergilli and Penicillia, and in particular *A. flavus*, was noteworthy. This organism is well-known for its ability to elaborate the dangerous aflatoxins.

In a study by Morey et al. (1984) it was observed that damp or water damaged areas in the home were particularly prone to fungal attack. In homes damaged by floods, fungal growth was found to be profuse in the indoor environment, with densities of up to 10200 CFU/m^3 recorded in flooded areas, 3000 CFU/m^3 in non-flooded areas, and 1290 CFU/m^3 in outdoor air. We noticed in this study that damp and water damaged buildings recorded the highest levels of surface mould growth as well as CFU/m^3 levels.

The close proximity of some of the homes to dumps was a concern to us since this would serve as a source of bioaerosols in the outdoor environment. Outdoor wind currents would then transport these facilitating their entry into the homes (Seltzer, 1994). Indoor environments lacking adequate ventilation, however, result in conidial densities well in excess of outdoor air (Mishra et al. 1992). Likewise, environments lacking wind currents primarily contain fungi with small conidia which commonly invade the human body through inhalation. It was therefore, no surprise that we found some *Aspergilli* and *Penicillia* predominating in these homes.

Although the study was carried out during the winter period, the higher indoor temperatures in the study homes would result in an indoor/outdoor pressure differential that would cause cooler air form the outdoor environment to be sucked indoors and in this way would carry with it several contaminants and fungal conidia in the air. Some of the homes with poor ventilation had dark interiors. This is known to contribute to increasing fungal growth, while high relative humidity levels provide fungi with one of its major nutritional requirements - water. Moreover, indoor substrates, which include plaster boards, wall paper, plastics, rubber and particularly wood, serve as ideal sites for fungal growth and proliferation. Wood provides compounds like sucrose, glucose and fructose encouraging profuse growth, however, when grown on inorganic building material, a nutrient deficiency stimulates the production of a number of volatile secondary metabolites (Bjurman & Kristensson, 1992). Since investigators still have not established whether the major threat is posed by the airborne conidia themselves or the mycotoxins they produce, one is able to appreciate the danger of promoting fungal proliferation in the indoor environments especially if the home is constructed from the afore-mentioned building materials.

This study has not investigated the ways in which the occupants of these homes attempt to alleviate mouldiness in their buildings. However, it has provided a basic information which will be useful for planning further studies in these communities.

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EVALUATION OF EXPOSURE TO ENVIRONMENTAL BACTERIA

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ABSTRACT

The present study provides information about the methods used to determine airborne bacteria in occupational environments. The best means proved to be the analysis of filter samples with endotoxin and peptidoglycan assays. The results of the assay, which measures the biological activity of cell wall components, mainly endotoxins, correlated well with the measured concentrations of viable airborne bacteria, especially with the levels of gram-negative bacteria. The endotoxin analyzed by chemical markers did not correlate well with the results of the assay nor with the viable bacteria levels, despite the similarity between the bacterial species identified from the culture media and the corresponding 3-OH fatty acids analyzed by the GC-MS assay. Indicative information on total bacteria levels could be obtained from peptidoglycans, which correlated well with total viable bacteria.

KEY WORDS

Bacteria, determination, endotoxin, exposure, environment, peptidoglycan

INTRODUCTION

Experimental studies on humans and other species have indicated that the exposure to high levels of inhaled bacteria and their cell wall components, such as the endotoxins of gram-negative bacteria, may injure the airways and cause many other health effects (Lacey and Dutkiewicz 1994, Zhiping et al. 1997). However, in epidemiologic studies the relationship between bacteria and health effects has been difficult to establish even though the symptoms of the persons studied have been typical of bacterial exposure. There may be several explanations for the failure to estimate a dose-response in epidemiologic studies. One may be the misclassification of exposure by the imprecise methods used to determine environmental bacteria levels because it is not clear which microbial bioaerosol components should be assessed (Eduard and Heederik, 1998).

Most determination methods have been developed for sampling and analyzing viable (culturable) bacteria. However, in occupational environments workers are exposed to bacteria via inhalation, and the bacteria, especially those in the air, are complex mixtures of viable and nonviable bacteria and their cellular components, such as endotoxins and peptidoglycans. The present study compares the suitability of some determination methods in the evaluation of exposure to environmental airborne bacteria at workplaces.

MATERIALS AND METHODS

The study was conducted in such occupational environments as slaughterhouses, silos, animal-feed processing plants, wood processing plants, a cotton yarn factory, a printing company and wastewater treatment and garbage handling plants. Both personal and stationary air samples were collected with sterile 37-mm diameter filters using calibrated suction pumps (Model 224, SKC Inc., Eighty Four, USA) at a flow rate of 2 l/min. Air sampling through glass fiber filters (85/220, Macherey-Nagel, Duren, Germany) was used to characterize the exposure to endotoxins and peptidoglycans. Polycarbonate membrane filters (0.2 μm pore; Nuclepore Corp., Pleasanton, USA) were used to collect bacteria. Viable bacteria were also collected from the air with a 6-stage impactor (Model 10-800 Andersen Inc., Atlanta, USA) on EMB and on R2A agar plates (Difco Laboratories, Detroit, USA) at a flow rate of 28.3 L/min. After the collection, the agar plates of total viable bacteria (R2A) were incubated at 20°C for 7 days, and agar plates of gram-negative bacteria (EMB) were incubated at 37°C for 48 hours. After the incubation the colonies were counted and the number of colony-forming units was determined according to the positive-hole correction of the Andersen impactor. Bacterial strains were differentiated on the basis of colony morphology and Gram's staining. Gram-negative bacteria were identified by API Systems test kits 20E for enterobacteria and 20NE for non-enterobacteria (bioMeriéux, Marcy-l'Etoile, France).

After the collection, the filters for the endotoxin and peptidoglycan assays were extracted with non-pyrogenic water, and the extraction solution was divided into two parts. From one part, the biologically active endotoxin concentration was determined with the endpoint chromogenic assay (Coatest® Endotoxin Test Kit, Chromogenix, Mölndal, Sweden). From the other, the total endotoxin level was evaluated using chemical markers (3-OH fatty acids of lipid A in endotoxin membrane fragments) as analytes in gas chromatography-mass spectrometry (GC-MS) according to Mielniczuk et al. (1992). The sample was lyophilized, hydrolyzed with strong acid, subjected to extraction and derivatization for the determination of the methyl-trimethylsilyl derivates of 3-OH acids using GC-MS with electron impact ionization. Peptidoglycans were determined from the same sample as the total endotoxin level using muramic acid as an analyte in a GC-MS assay. The muramic acid of peptidoglycan was analyzed as a trifluoroacetylated methyl glycoside using GC-MS in combination with chemical ionization according to Elmroth et al. (1993).

The filter samples for the bacteria assay were diluted to various concentrations after extraction with a 0.1% peptone solution with 0.01% Tween-80, and culturable bacteria levels were determined by plating 0.1 ml of the extraction solution onto EMB and R2A media. The agar plates were incubated with the same manner as the plates after collection with the impactor. After the samples were removed for the endotoxin analyses by the *Limulus* assay and the culturable bacteria analyses, the bacterial cells were stained with acridine orange so that the total bacterial counts could be calculate by epifluorescence microscopy (Laitinen et al. 1992).

RESULTS AND DISCUSSION

DETERMINATIONS OF CULTURABLE AND NON-CULTURABLE BACTERIA

In Finland the Andersen impactor has been the most commonly used for collecting environmental bacteria from the air, although the selection of culture medium can affect the results. EMB culture medium has been found to collect the highest counts when compared with other gram-negative-bacteria-selective media, and its selectivity for gram-negative bacteria is 84%, on average (Laitinen et al. 1992). The comparison was performed using samples from wastewater treatment plants, but EMB proved to be suitable for the sampling of gram-negative bacteria also in other environments (Table I). A high correlation between bacteria concentrations on EMB and the endotoxin concentrations of gram-negative bacteria in the *Limulus* assay was found for 8 of 10 various occupational environments.

TABLE I: Number of occupational environments for which the correlation coefficient (r) was ≥ 0.6 between methods of determining bacteria levels.

	LAL & EMB	LAL & R2A	LAL & GC-MS	GC-MS & EMB	GC-MS & R2A
Number of workplaces	8/10	6/10	6/13	5/10	3/10

LAL = assay, GC-MS = an assay for 3-OH fatty acids of endotoxin

The filter sampling for culturable and non-culturable bacteria was not found to be useful. Dilution plating after the extraction of the filter showed very few numbers of culturable bacteria because most of the bacteria probably desiccated during the sampling. The fluorescence microscopy assay for non-culturable bacteria involved technical problems with the bacteria calculations. The size of the bacterial cells is often under 5 μm, and therefore especially spherical bacteria are difficult to recognize from other stained particles, which are also present in filter samples. On the other hand, filter collection of bacteria was not suitable for the personal exposure measurements, because the subjects themselves emitted bacteria from their bodies into the air, and these bacteria were also collected on the filters. Therefore, the non-culturable bacteria results of the personal samples did not depend only on the work environment, as shown by the background levels of the personal samples which might be about 10^6 bacterial cells/m^3.

DETERMINATIONS OF BACTERIAL CELL WALL COMPONENTS, ENDOTOXIN AND PEPTIDOGLYCANS

The endotoxin results analyzed with the *Limulus* assay correlated more often with the viable bacteria concentrations than did the endotoxin results of the GC-MS (Table I). Therefore, the biological activity of endotoxins, which is analyzed in the *Limulus* assay, is dependent on the viable gram-negative bacteria levels in the sampling environment. Although for most of the workplaces, the GC-MS assay of endotoxin did not correlate well with the viable bacteria concentrations, the analyzed profiles of the 3-OH fatty acids corresponded to some of the bacterial species identified in the culture media. In the air of slaughterhouses and animal food production plants, members of the family *Enterobacteriaceae* were the most common bacteria, and the majority of 3-OH fatty acids were 3-OH-14:0 fatty acids. On the other hand, 3-OH-12:0

fatty acids were common in the wood processing plants where strains belonging to *Acinetobacter, Chryseomonas,* and *Pseudomonas* bacteria were identified from the air. However, the identification of bacterial species from from culture media was possible only for a small number of bacteria. Many other possible bacteria (e.g. anaerobics) do not grow on the selected culture media. This problem is illustrated by the fact that 3-OH-16:0 fatty acids were common at many workplaces although the corresponding culturable bacteria for them was less frequently found in the air samples.

No correlation was found at all between the results of the *Limulus* and GC-MS assays when the endotoxin results of all the workplaces were clustered together. However, the results of these analytical methods did correlate for some individual workplaces (Table I), the endotoxin air samples including mainly 3-OH-12:0 or 3-OH-14:0 fatty acids in the GC-MS assay. In their study of settled house dust samples Saraf et al. 1997. found that the type of 3-OH fatty acid in the endotoxin may play an important role in determining the extent of correlation between *Limulus* and GC-MS assays.

An unexpectedly high correlation ($r = 0.68$) was found between peptidoglycans and total culturable bacteria although the results of the workplaces were clustered together, and the peptidoglycans were analyzed as muramic acid in the GC-MS assay, which is not dependent on the viability of bacteria. The peptidoglycan differs from endotoxin in that all bacteria have peptidoglycan in their cell walls. The muramic acid content in the peptidoglycan membrane of gram-positive bacterial cells is commonly greater than that of gram-negative bacteria (Fox et al. 1993). Because gram-positive bacteria will not lose their viability as easily as gram-negative bacteria, the correlation between peptidoglycan and viable total bacteria may be easier to establish than that with viable bacteria and endotoxins analyzed by the GC-MS assay.

CONCLUSIONS

Exposure to airborne bacteria can be evaluated from the breathing zone of mobile persons from the endotoxins and peptidoglycans detected using filter sampling. These determination methods are suitable for assessing bacterial exposure also in high bacteria concentrations during a long period (e.g., an 8-hour workshift) because the filter sample does not overload. Because gram-negative bacteria easily lose their viability and culturability during air sampling, exposure to these bacteria is better evaluated through the detection of endotoxin with the *Limulus* assay than through the detection of viable bacteria with different culture media. On the other hand, the identification of gram-negative bacteria and gram-positive bacteria (except actinomycetes, which can obtained by culture-based methods) is not generally needed in the evaluation of exposure to non-pathogenic environmental bacteria. Peptidoglycan assay may be suitable for quantitatively determining total bacteria levels, but future studies are needed to adapt the methods used to analyze peptidoglycans as a routine assay.

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ANALYSIS OF MICROBIAL CONTAMINATION OF A VENTILATION SYSTEM DETECTED BY MEASUREMENT OF MICROBIAL VOLATILE ORGANIC COMPOUNDS

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INTRODUCTION

Measurement of atmospheric microbial volatile organic compounds (MVOCs) has been identified as a method to assess microbial growth in indoor environments. Complex mixtures of volatile organic compounds (VOCs) including alcohol, ketones, aldehydes, aromatic and chlorinated hydrocarbons, sulfur-based compounds, amines, and terpenes are known to be produced by microbial metabolism (Burge, 1995).

The measurement of atmospheric MVOCs has several potential advantages in identifying building microbial amplification sites compared to measurements of atmospheric fungi or bacteria. MVOC analysis may also serve as a sensitive marker of microbial growth. Periods of high mold spore production may be preceded by increased MVOC production (Abramson et al. 1983). MVOCs are gases and can readily disperse throughout a building and potentially penetrate barriers such as vinyl wallpaper and polyethylene sheeting (Strom, 1994). Such barriers may impede spore transport. MVOC analysis can potentially identify indoor microbial amplification sites before there are any visible signs of growth, especially if the growth is present in poorly accessible areas or areas that are not subject to direct inspection (Borjesson, 1990).

MVOC analysis has not been widely utilized in the assessment of building microbial problems for several reasons. The factors responsible for stimulating and modulating MVOC production and metabolism in fungi and bacteria have not been well characterized. Although fungal growth as measured by carbon dioxide production has been correlated with MVOCs, data on specific MVOC production associated with individual fungi and bacteria are scant (Borjesson, 1990).

The interpretation of MVOC results is difficult since there is limited information on concentrations that represent normal or typical indoor values. VOCs that have been identified as being produced by fungi and bacteria can also be generated by other biogenic sources including trees and algae. The other limiting factor is the concern that indoor MVOC concentration may not be related to other measures of fungal activity (Miller, 1988). MVOC testing is also more costly than many other tests commonly used to assess indoor microbial problems.

Using data from an office building in California, this study demonstrates that measurement of MVOCs can be an investigation tool to assess hidden bioaerosol sources when there is a high

index of suspicion of a source, and, especially, when other environmental tests fail to identify a problem.

A CASE OF AN OFFICE BUILDING IN CALIFORNIA

BACKGROUND

This study involved a six-story municipal government facility built in 1970 and located in Southern California. The building remained problem-free until the HVAC system was reengineered and installed in December 1995. The modifications included replacing an old 700-ton chiller with a high efficiency 400-ton unit. A new cooling tower with an automated water treatment chemical feed system to control bacterial growth was installed. Separate HVAC systems supplied the west and east sections of the building. The modifications resulted in more than a 20% increase in energy savings and the building won the 1997 ASHRAE (American Society for Heating, Ventilation and Air Conditioning Engineers) Award for application of innovative technology in the governmental sector.

Odor-related events described as being chlorine-like began occurring by mid January 1996 in the west section of the 5th floor. Occupants of the east section reported no complaints. Subsequent investigations revealed that the cooling tower water located near the HVAC intake for the west section of the 5th floor was excessively chlorinated. Symptoms reported by occupants primarily included upper respiratory tract and eye irritation. The occupants also reported a general fear of toxic gas exposure and the building was evacuated on several occasions. Testing by local hazardous materials teams using direct reading instruments revealed no significant chlorine levels. Complaints among occupants of the west section of the 5th floor continued. Other problems reported included headaches and fatigue, and stuffy and musty overall air quality.

The building underwent several environmental investigations by different consultants over approximately two years. In early 1996, tests for indoor carbon dioxide (CO_2) revealed concentrations ranging from 600 to 900 parts per million (ppm). This finding is consistent with adequate outdoor fresh air supply. In April 1996 tests for VOCs using a modified US EPA TO-14 method revealed low concentrations of several compounds. The maximum concentrations of the principal compounds were Freon-22 at 41.5 parts per billion (ppb) (136 ug/m^3) and toluene at 6.8 ppb (26 ug/m^3). In December 1996, an inspection of the HVAC system including the variable air volume (VAV) control boxes revealed no problems. Microbial tests including scotch tape samples from the supply ducts and cultures of dust collected from the VAV boxes were interpreted as revealing normal background microbial flora.

Investigations in 1997 included continuous monitoring of carbon monoxide (CO), CO_2, temperature and relative humidity. These parameters met ASHRAE environmental and air quality recommendations (ASHRAE, 1989). Repeat VOC testing revealed low concentrations of several compounds which were considered typical of an office environment. An observation was made that misting from the cooling tower serving the west section of the 5th floor could be entrained into the HVAC system during windy days due to the close proximity of the cooling

tower to the HVAC. However, the HVAC components, including the cooling coils, drain pans, and visible duct liner, appeared dry and showed no visible evidence of microbial growth.

Employees were referred to a university-based occupational and environmental medical clinic. Their reported symptoms were considered to be consistent with a bioaerosol exposure problem. The reported symptoms provided a strong impetus to continue building investigation, although past environmental studies had failed to identify any definite findings. Based upon the symptom pattern reported, which suggested allergy-like reactions, further detailed microbial testing was conducted in the building. The results and observations are described below.

METHODS

Several areas of the building were selected for study. This included the problem area on the west section of the 5th floor, a non-problem area on the same floor (east section supplied by the other HVAC), and a separate control floor. All air samples were taken in adult breathing zones with the exception of the MVOCs, which were taken immediately beneath the air supply ducts. Several duplicate air samples were collected for viable fungi.

Viable mold and bacteria samples were collected using a single stage Andersen sampler. Rose bengal and czaepek cellulose agar were used to culture fungi. Environmental bacteria collected on R2AC Agar and cultured at room temperature. Human commensal organisms were collected on blood agar and cultured at 35ªC. Thermophyllic bacteria were collected on tryptic soy agar and cultured at 56ªC. The analyses were performed by Pathcon Laboratories, Norcross Georgia. Total spores were collected onto a slit impactor (Zefon filter). The analysis was conducted by P and K Microbiology Services, Inc., Cherry Hill, New Jersey.

MVOC samples were collected onto Anasorb 747 (SKC Inc.) sorbent tubes for 243 to 317 minutes at a flow rate of approximately 0.2 liters per minute. The tubes were chemically desorbed and analyzed using GC-MS analysis operated in selected ion mode (SIM). This method increases the sensitivity to MVOCs. The detection limit was 0.0005 ug or approximately 0.01 ug/m^3 for a 48-liter sample. Compounds detected include 3-methylfuran, 1-octen-3-ol, 3-octanone, 3-octanol, 3-heptanone, 2-methoxy-3-(1-methylethyl) pyrazine, 3-methyl-2-butanol, 2-pentanol, 3-methyl-1-butanol, 2-hexanone, 2-methylisoborneol, geosim, and 2-octen-1-ol. The analyses were conducted by Air Quality Sciences, Atlanta Georgia.

Surface dusts were collected from inside the building. These bulk samples were collected using the Microvacuum method. Surfaces included horizontal shelving and wood door frames. Bulk samples were also collected of the HVAC liner. Scotch tape was also used to collect surface samples for microscopic evaluation. The bulk and surface samples were cultured using MEA media by P and K Microbiology Services, Inc., Cherrry Hill, New Jersey. Tryptic soy agar was used for bacterial culture of the HVAC duct.

Surface dusts from inside the building were also submitted for particle characterization by manual scanning electron microscopy (MSEM). This was performed by RJ Lee Group, Inc., San Leandro, California.

OBSERVATIONS AND TESTING RESULTS

Observations of the cooling tower revealed that it was located within 30 feet of the HVAC intake on a rooftop deck. The HVAC ducts, including areas that were difficult to reach, were inspected. The duct liner near the intake appeared to have no mold growth, but a more extensive evaluation of the liner revealed areas of visible brown colored powdery-like growth which was later found to affect significant portions of the interior of the ducts supplying the west section of the 5th floor. Other areas had white staining and there was evidence of glue-like residue inside the duct.

TABLE I: Atmospheric Concentrations of Fungi.

Location	Total spores s/m³*	Predominate fungi (Percentage)	Viable fungi cfu/m³** (range)	Predominate fungi (Percentage)
Ambient	6658	*Cladosporium* (75) Unknown(10) *Penicillium/Aspergillus* (5)	389 and 506	*Cladosporium* (70) Sterilia mycelia (16) *Aspergillus niger* (5)
Complaint Area: West 5th Floor (Site 1)	914	Unknown (37) *Cladosporium* (26) Drechslera/Bipolaris (6)	35 and 71	Unidentified fungi (67) *Aureobasidium* (17) *Penicillium* (17)
Complaint Area: West 5th Floor (Site 2)	418	Unknown (25) *Cladosporium* (19) *Penicillium/Aspergillus* (6)	NSC***	NSC***
Complaint Area: West 5th Floor (Site 3)	131	*Penicillium/Aspergillus* (60) Ascospores (20) Unknown (20)	35	Unidentified fungi (67) Yeast (33)
Non-Complaint Area:East 5th Floor	253	*Alternaria* (21) *Cladosporium* (10) Unknown (10)	12	*Cladosporium* (100)
Other Floor	287	Unknown (36) *Cladosporium* (27) *Penicillium/Aspergillus* (9)	24	*Cladosporium* (50) Sterila Mycelia (50)

Notes: * s/m³: Fungal structures per cubic meter.
** cfu/m³: Colony forming units per cubic meter.
*** NSC: No sample collected.

The tests for airborne fungi revealed that the concentrations of total and viable molds were very low and considerably below concentrations found outdoors. These results are described in Table 1. The average concentration of total spores inside was 401 structures per cubic meter (s/m³) and ranged from 131 to 914 s/m³. The ambient concentration was 6,658 s/m³. The predominant fungi inside in most sites were unknown spores and *Cladosporium*. *Cladosporium* and unknown spores were also the main fungi outdoors. Small spores characteristic of *Penicillium*, *Aspergillus spp,* and *Alternaria* were the main fungi at two sites indoors in both problem and non-problem areas. The average viable fungal concentrations were very low. The average concentration was 34 colony forming units per cubic meter (cfu/m³) and the individual values ranged from 12 to 71 cfu/m³. The level outdoors was 448 cfu/m³. Predominant organisms inside

included „unidentified fungi", *Cladosporium* and sterile mycelia. *Cladosporium* and sterile mycelia were predominant outdoors.

TABLE II: Microbial Volatile Organic Compound (MVOC) Concentrations.

Location	**MVOC Concentrations Initial testing (ug/m³)***	**MVOC concentration Follow-up testing (ug/m³)**
Ambient	Total: 2.0 3-Octanone: 2.0	Total: 3.4 3-Methyl-1-butanol: 1.4 2-Hexanone: 0.8 2-Heptanone: 1.1
Complaint Area: West 5th Floor (Site 1)	Total: 31.7 2-Pentanol: 1.4 3-Methyl-1-butanol: 1.5 2-Hexanone: 8.8 2-Octen-1-ol: 12.7 1-Octen-3-ol: 7.4	Total: 15.6 3-Methyl-1-butanol: 7.7 2-Hexanone: 2.1 2-Heptanone: 1.1 2-Octen-1-ol: 2.8 1-Octen-3-ol: 1.9
Complaint Area: West 5th Floor (Site 2)	Total: 12.9 3-Methyl-1-butanol: 1.5 2-Hexanone: 6.8 2-Octen-1-ol: 3.2 3-Octanol: 1.3	Total: 10.0 3-Methyl-1-butanol: 3.1 2-Hexanone: 0.8 2-Heptanone: 1.1 2-Octen-1-ol: 2.9 1-Octen-3-ol: 2.0
Complaint Area: West 5th Floor (Site 3)	Total: 20.7 3-Methyl-1-butanol: 1.5 2-Hexanone: 8.0 2-Octen-1-ol: 6.3 3-Octanone: 1.6 1-Octen-3-ol: 3.2	NSC***
Non-Complaint Area: East 5th Floor	Total: 30.1 2-Pentanol: 1.7 3-Methyl-1-butanol: 1.8 2-Hexanone: 2.9 2-Octen-1-ol: 13.1 3-Octanone: 3.0 1-Octen-3-ol: 7.0	Total: 13.1 3-Methyl-1-butanol: 8.7 2-Hexanone: 1.1 2-Heptanone: 1.2 1-Octen-3-ol: 2.1

Note: **ug/m³: Microgram per cubic meters of air.
*** NSC: No sample collected.

Tests for atmospheric bacteria did not reveal unusual concentrations or uncommon species. Concentrations of environmental bacteria (cultured at room temperature) ranged from 85 to 236 cfu/m³, while the outdoor levels ranged from 648 to 730 cfu/m³. The main bacteria inside were *Staphylococcus* (not *aureus), Bacillus Arthrobacter*-like and gram negative rods, while *Acinetobacter* and *Athrobacter*-like species were predominant outdoors. Human commensal organism concentrations ranged from 94 to 165 cfu/m³; outdoors the levels ranged from 236 to 448 cfu/m³. The main bacteria inside were Staphylococcus species (not aureus) and *Bacillus* species while outdoors *Corynebacterium*-like and *Brevibacterium*-like bacteria were predominant. Insignificant levels of *Thermophilic* species were measured inside and outdoors.

Repeat tests for atmospheric fungi were conducted after the HVAC intake for the west section near the cooling tower was sealed to stop moisture intrusion. Follow-up testing revealed low concentrations of fungi inside. The average concentrations of total spores inside was 853 s/m^3 and ranged from 653 to 1123 s/m^3. The outside concentration was 2, 298 s/m^3. The predominant organism inside and outdoors was *Cladosporium*. Sampling for viable fungi revealed that the average concentration inside was 106 cfu/m^3 and ranged from 24 to 212 cfu/m^3. The ambient concentrations ranged from 542 to 754 cfu/m^3. *Cladosporium* was the main organism outdoors with the exception of two areas where sterile mycelia were predominant.

MVOC testing results are presented in Table II. Indoor MVOC concentrations were much higher than outdoors and ranged from 12.9 to 31.7 ug/m^3. The principal compounds were 2-hexanone, 2-octen-1-ol, and 1-octen-3-ol. Outdoors, 3-octanone was detected at a concentration of 2.0 ug/m^3. Repeat studies conducted after the west section HVAC was sealed revealed decreased total MVOC concentrations in all areas. 2-octen-1-ol and 1-octen-3-ol decreased while the concentration of 3-methyl-1-butanaol increased.

Several analyses were conducted of surface dusts collected from inside the building. Scotch tape samples from horizontal surfaces revealed counts range from 4 to 10 spores. Table III presents the results of fungal cultures of suspended dust. The concentrations and species in complaint areas did not differ substantially from those of other areas. The main fungi were *Cladosporium, Rhodotorula, Penicillium*, and *Alternaria*.

TABLE III: Concentration of viable fungi in suspended dust collected from building interior.

Sample description	Surface Total Concentration (CFU/g)*	Fungal (%)
Complaint Area: West 5th Floor (Site 1)	85,714	*Rhodotorula* (26) *Cladosporium* (19) *Aspergillus sydowii* (17)
Complaint Area: West 5th Floor (Site 2)	75,780	*Cladosporium* (64) *Pennicillium* (12) *Aspergillus niger* & *ochraceus* (8)
Non-complaint Area: East 5th Floor	86,364	*Alternaria* (42) *Cladosporium* (37)
Other Floor	18,182	*Rhodotorula* (65) *Cladosporium* (15)

Note: *Colony forming units per gram of material.

Composite dust samples from inside were also analyzed by MSEM. Surface dust was collected from shelving, window frames, picture frames, and doorway frames from problem and non-problem areas. The majority of the dust collected in both samples was earth crust material (soil) and carbonaceous material. The earth crustal material was primarily Si/Al/Mg-rich material. The carbonaceous material consisted of skin flakes and, to a lesser degree, animal dander

and fragments of cellulose material. Moderate amounts of iron-rich particles were seen that may have originated from the HVAC system. Trace amounts of cellulose and biological fibers of approximately 100 um in length were seen that could be insect or plant fragments. There was no evidence of significant concentrations of fiberglass or mold spores.

The results of the analysis of bulk samples of the HVAC duct liner are presented in Table IV. Duct liner samples that appeared to have brown powder-like discoloration consistent with mold growth revealed nearly 100% growth of *Cladosporium* at concentrations of 2,250,981 cfu/g. The specific species was not identified in this analysis. Duct liner samples from an adjacent area that did not have visible mold growth had a concentration of *Rhodotorula* and yeast at 3,698,039 cfu/g and a bacterial concentration of 25,403,920 cfu/g. *Methylobacterium* and other gram-negative organisms were the primary organisms. The duct lining had dried glue-like material that had a total fungi concentration of 6,609,483 cfu/g, and the main organisms were *Cladosporium* and *Rhodotorula*. The concentration of bacteria from a sample of smeared glue was 3,075,000 cfu/g, and Methylbacterium was the principal bacteria. The duct liner was removed and repeat tests of new material revealed that the concentration of spores from duct work was less than 100 cfu/g.

TABLE IV: HVAC Fibrous Duct Liner Culture.

Sample Description	**Bacteria Species (CFU/g)***	**Fungal Species (CFU/g)**
Powder-like "growth"	Bacteria / 980 Total Bacteria: 980	*Cladosporium*/ 2,250,981 Total Fungi: 2,250,981
Composite sample from several areas affected by the same brown "growth"	Bacteria/ 952 Total Bacteria: 952	*Cladosporium*/ 4,334,286 Total Fungi: 4,334,286
White, "dry-glue" like smear	gram (-) bacteria & others/ 494,838 Methylobacterium/ 2,2580,173 Total Bacteria: 3,075,000	*Cladosporium*/ 3,675,862 *Rhodotorula*/ 2,050,000 *Tilletiopsisi*-like yeast/ 424,138 Total Fungi: 6,609,483
Possibly water damaged fibrous liner, white-colored stain	Flavobacterium: 624,760 Gram (-) bacteria & others: 507,619 Methylobacterium: 1,327,619 Rhodococcus: 546,667 Total Bacteria: 3,006,667	*Aureobasiduim pullulans*/ 78,095, *Rhodotorula*/ 1,835,476, Yeast/ 2,030,476 Total Fungi: 3,943,810
Relatively clean and unstained	Gram (-) bacteria & others / 10,290,200	*Rhodotorula*/ 1,366,667 *Tilletiopsisi*-like yeast / 683,333 Total Fungi: 3,698,039

Note: *Colony forming units per gram of bulk material.

DISCUSSION AND CONCLUSION

MVOC analysis can be considered an investigative tool to assess hidden microbial amplification sites when there is a high index of suspicion of bioaerosol problems, especially when other environmental tests fail to identify an amplification site. In this study, tests commonly used to assess microbial contamination, including airborne total and viable fungi and bacteria, revealed low concentrations. Extensive fungal growth was subsequently observed in the air supply ducts that had not been identified in prior building investigations.

The average concentration of total spores inside this building was 329 s/m^3 (ranging from 287 to 914 s/m^3), while the outdoor level was much higher (6658 s/m^3). This low indoor concentration compared to outdoors is generally considered to be consistent with findings in non-problem commercial buildings. The concentrations of viable fungi inside the building were also very low. The low concentrations would likely be considered by many investigators as not being indicative of an amplification site even though there were some differences between fungi inside the building and those outdoors. The viable culture testing did not differentiate the species of *Cladosporium* and the possibility exists that the type of *Cladosporium* detected outdoors differed from the species indoors. Determining the type of *Cladosporium* species found can provide valuable information for determining an amplification site. Testing for airborne bacteria did not reveal unusual concentrations or species inside the building.

Analysis of suspended dust from inside the building surfaces also did not suggest a definite amplification site. The ratio of fungal colony counts of all species to the sum of colony counts of *Cladosporium, Alternaria*, and *Epicoccum spp* was less than one order of magnitude. If this ratio is one order of magnitude or greater fungal growth in a building is considered likely (Dillon et al. 1996). Findings of dust collected by scotch tape revealed similar results in problem and non-problem area. Electron microscopy analysis of dust collected from two areas revealed it was composed of earth crust materials or soils and there was no evidence of unusual loading of mold spores or materials.

The concentrations of MVOCs inside the building were much higher than outdoors. Total MVOC concentrations decreased with moisture control measures indicating that the measured levels were most probably related to mold growth. No concentration of 2-octen-1-ol, considered to be an indicator of old growth, diminished inside the building after the introduction of moisture control measures. The concentration 3-methyl-1-butanol increased.

The MVOC test results coupled with occupant complaints provided a strong impetus to conduct further investigations, including inspecting poorly accessible areas of the air supply ducts, to identify an amplification site. MVOC testing, however, should not be considered as a routine test because limited information exists regarding the significance of measured MVOC concentrations and the normal range of concentrations in non-problem buildings.

The published study with the largest number of MVOC samples revealed that the average total MVOC concentrations in 119 air samples was 50.5 ug/m^3 (Morey et al. 1997). The most prevalent compounds were 2-octen-1-ol and 1-octen-3-ol which were found in more than 30% of samples at average concentrations of 16.1 ug/m^3 and 4.8ug/m^3, respectively. The average outdoor concentration was 6.5 ug/m^3 (20 samples). However, no information on the number of buildings tested, the building conditions or the extent of microbial growth was available for

most of these samples. Data on four buildings with known mold problems revealed increased MVOC concentrations indoors compared to outside, even though tests for viable fungi revealed no unusual concentrations.

One large study of 52 Canadian homes of which seventy percent had air quality problems suggested that there is little relationship between indoor total MVOC concentrations and other measures of fungal activity (Miller, 1988). The total VOC concentrations ranged from 80 to 1,890 ug/m^3 (mean of 680 ug/m^3). Three VOCs were considered as likely to have originated from microbial sources (3-methyl-1-butonal, 2-hexanone, and 2-heptanone). However, the concentrations of these specific compounds were not provided.

There are differences in average concentrations of MVOCs among several published studies. The concentrations measured in this study are similar to those reported by Morey et al. (1997) which evaluated problem buildings, but much lower than the study of Canadian homes (Miller, 1998). These differences in concentrations may reflect methodological differences in performing the tests and the range of compounds identified as considered likely to have originated from microbial sources. This information needs to be considered in the evaluation of MVOC data from any building.

The other factor limiting the interpretability of MVOC data is the lack of source specificity for many compounds. Compounds which have been identified as originating from microbial sources can have other sources. For example, 2-hexanone is used in lacquers, resins, acrylates and other coatings (HSDB, 1998).

This study highlights that even though the redesign of the HVAC system resulted in improved energy performance, the retrofit did not include relocation of a cooling tower which was located within 30 feet of the west HVAC air intake located in a rooftop courtyard area. The chlorination system was changed but not the location. Health concerns should be considered in building redesign in addition to energy efficiency.

The source of the occupant complaints cannot be attributed to the MVOC concentrations alone. Other areas of the building including a non-complaint area on the 5th floor served by another HVAC and another floor had similar concentrations. This may be due to diffusion of MVOCs through the building into areas served by an HVAC not impacted.

Further research is needed in the areas on normal MVOC ambient and indoor concentrations, factors responsible for growth, and specificity of compounds in relationship to growth of fungi and bacteria to further assess the applicability of MVOC measurement in the evaluation of problem buildings.

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MYCOBACTERIA AND RELATED GENERA ARE MAJOR COLONIZERS OF A WALL IN A CHILDREN'S DAY CARE CENTER

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ABSTRACT

We quantitated and identified the cultivatable CMN (Nocardioform actinomycetes) taxons in water damaged and undamaged gypsum liners from an indoor wall in a children's day care center. The bacteria were characterized chemotaxonomically and their 16S rDNA sequenced. In the water damaged site most frequently isolated bacteria were CMN taxons; *Dietzia sp* (10^5 cfu g^{-1}) and a new rapidly growing *Mycobacterium* species, *Mycobacterium murale sp. nov*[1], (10^6 cfu g^{-1}). *M. murale* was found to be closely related to the pathogen *M. abscessus*. One of *M. murale* strains was able to grow on toluene which is contained in commonly used cleaning agents in day care centers. The only CMN taxon found in the non water damaged liner was representative of a new genus, *Williamsia muralis gen. nov. sp. nov.*. (10^3 cfu g^{-1}). Environmental mycobacteria, related to pathogenic species may survive and proliferate in water damaged indoor building materials.

KEY WORDS:

Nocardioform actinomycetes, *Mycobacteria*, indoor contaminant, indoor building materials

INTRODUCTION

Indoor building materials exposed to repeated water damages with intervening dry periods represent favorable habitats for desiccation resistant bacteria like actinomycetes. The mycolic acids containing actinomycetes, also called the Nocardioform actinomycetes or the CMN taxons includes the families *Corynebacteriaceae, Mycobacteriaceae* and *Nocardiacea* (Chun et al. 1996). The cell wall components of these taxons are known as stimulators of the immune system. Exposure to aerosolized cell fragments may be a health hazard (Ikeda-Fujita et al. 1987, Ridell 1983, Shoenfield and Isenberg 1988). Environmental Nocardioform actinomycetes like *Gordona, Nocardia*, and *Mycobacteria* include species ranging from harmless inhabitants of water and soil to pathogenic species (Guay 1996). In Finland, atypical mycobacteria have become more frequent in clinical samples, but their reservoir in the environment has not been identified (Katila et al. 1995). There are a few reports of mycobacteria in indoor dusts and air (Flannigan, 1995, Reznimov et al. 1971, Tsukamura et al. 1974) and tap water (Peters et

al.1995), but the occurrence and taxonomy of Mycobacteria and other Nocardioform actinomycetes in indoor environment as well as their proliferation and survival in building materials has not been investigated. We quantitated and identified the cultivatable actinomycetes, focusing on the CMN taxons, in indoor building materials and dust in a children's day care center.

MATERIALS AND METHODS

Isolation and quantitation of aerobic heterotrophic bacteria:

The bacteria were sampled from both water damaged and non damaged gypsum liners used as indoor wall material and also from settled dust of a children's day care center with a 10 year history of repeated water damages. The water damaged gypsum liner was sampled from the wall close to floor level where evidence of damage was visible as change in colour and conspicuous microbial growth. Before cultivation the samples were air-dried for 2 weeks. The bacteria were resuscitated (Andersson et al. 1995) and viable bacteria cultured on tryptic soy agar at 16°C and 22°C as described in Andersson et al. (1997).

Identification of bacteria:

Bacterial isolates were identified according to whole cell fatty acid composition, 16S rDNA sequencing and chemotaxonomical characterization as described elsewhere (Andersson et al. 1997, Andersson et al. 1998, Vuorio et al. 1999, Kämpfer et al. 1999).

Toluene utilization:

The ability of the strains to utilize toluene as a source of carbon and energy was tested in batch culture with KSN mineral medium (Zaitsev et al. 1995). Bacteria were incubated at 28°C in 500-ml Teflon-stoppered flasks with 100 ml of culture. Toluene was supplied as vapor and also added directly to the medium at concentration of 20 ppm. Growth was monitored by measuring the optical density at 540 nm. Inoculated cultures with no toluene were used as control.

Microscopic analyses:

Building materials and cells from bacterial strains were analyzed by transmission electron microscope as described in Andersson et al. 1997.

RESULTS

Figure IA shows that the water damaged gypsum liner contained a complex microbial community including bacteria and fungi. Thin sections of the water damaged gypsum liner (Figures 1B and 1C) show bacterial cells with a cell division pattern and cell walls characteristic for actinomycetes.

The water damaged gypsum liner contained 10^7 cfu g^{-1} and the non water damaged gypsum liner contained 10^4 cfu of cultivatable aerobic heterotrophic bacteria g^{-1}. The identified species of actinomycetes isolated from the indoor environment of a children's day care center are shown in Table I. From the water damaged gypsum liner 48 strains representing 17 different

FIGURE 1: Thin sections of water damaged gypsum liner. Panel A shows a complex microbial community of bacteria and fungi inhabiting the gypsum liner. Bar is 1000 nm. Panels B and C show thin sections of bacterial cells in the gypsum liner. The figures show cell wall structures and cell division typical for actinomycetes. Bars are 500 nm (B) and 200 nm (C).

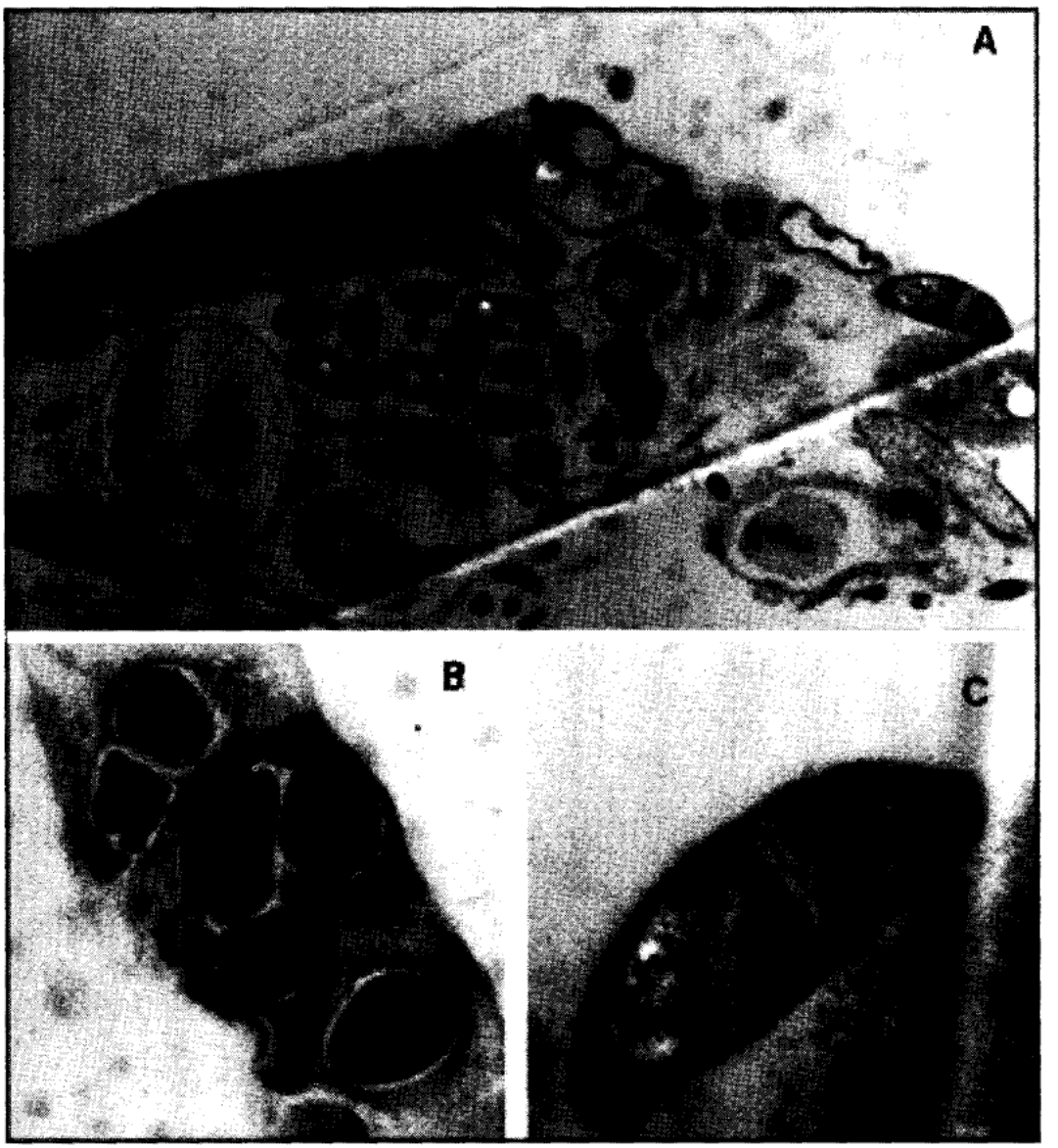
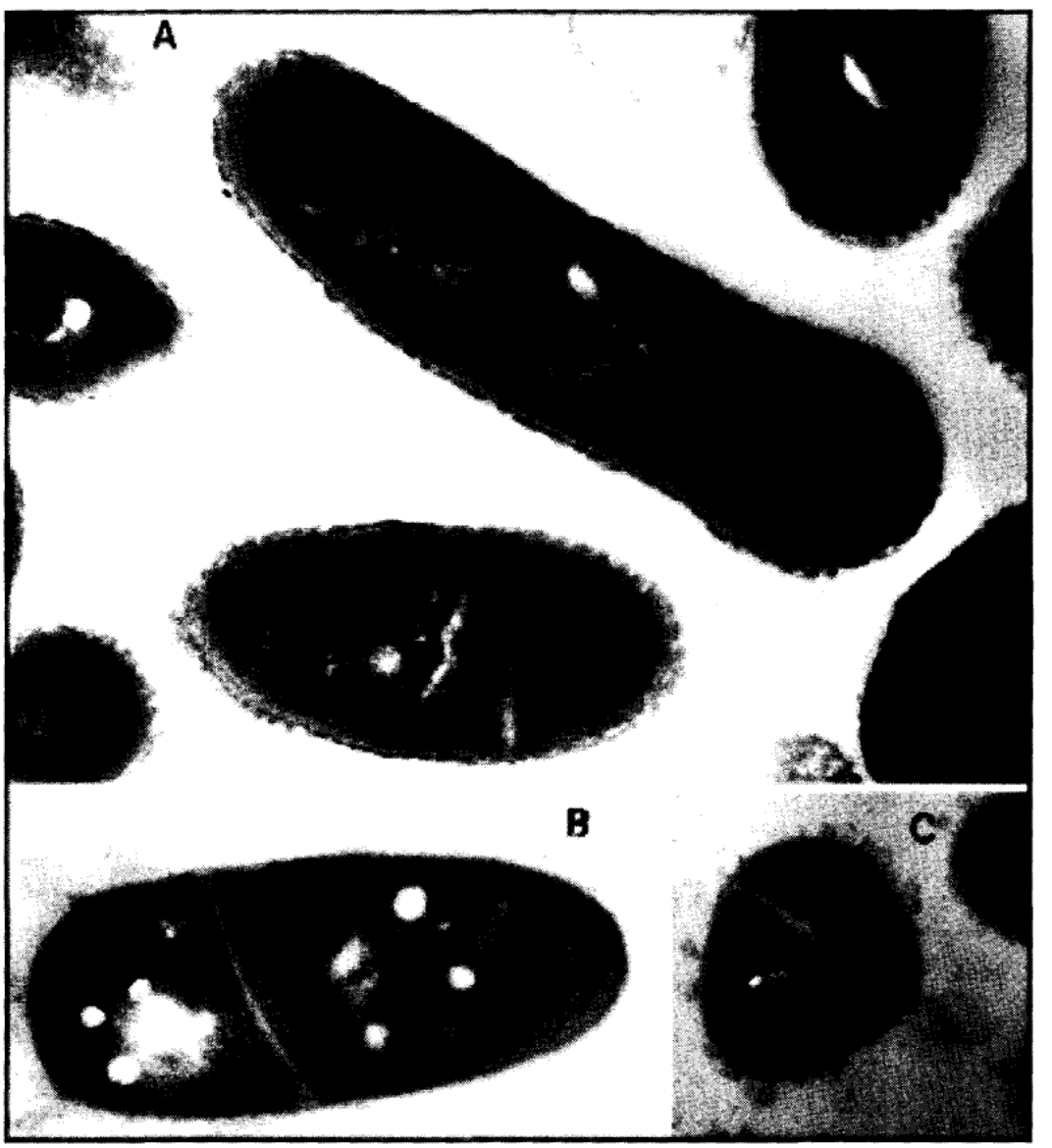


FIGURE II: Thin sections of bacterial cells of the CMN taxons found in gypsum liners from indoor walls from a children's day care center. Cells were grown for 7 d on tryptic soy agar at 28°C. Panel A shows *Mycobacterium murale sp. nov.*, strain MA-112, exhibiting a triple layered structure of the cell wall. The picture shows an irregular electron dense outer layer, an electron translucent layer and a peptidoglycan layer. Panel B shows *Williamsia muralis sp. nov.*, strain MA-140. The capsule surrounding the cell wall is visible as hairy appendages in the thin section. The cell wall of *Dietzia sp.*, strain MA-148 is shown in Panel C. Also these cells seems to be surrounded by a capsule. Bars are 200 nm.



taxons were identified. Eight bacterial strains represented CMN taxons: two strains of *Dietzia* sp. (10^5 cfu g^{-1}) and six strains of a rapidly growing scotochromogenic mycobacterium identified as a new species, *Mycobacterium murale sp. nov*[1], (10^6 cfu g^{-1}). The other 40 strains represented 15 different taxons. From the non water damaged gypsum liner, 15 strains representing 8 different taxons were isolated and identified. The only CMN taxon found (one strain) was a new genus tentatively named *Williamsia muralis gen. nov. sp. nov.* (10^3 cfu g^{-1}). These results show that *Mycobacteria* and *Dietzia* were among the most frequently isolated taxons and occurred in high densities in the water damaged gypsum liner. *Mycobacteria* and *Dietzia* were not detected in the undamaged part of the liner above the detection limit of 10^3 cfu g^{-1}. Thin sections exposing the different cell wall structures of *M. murale, Dietzia sp.* and *Williamsia muralis* are shown in Figure II.

TABLE I: Actinomycetes isolated from a children's day care center.

Name	Density	Health hazard	Habitat	References
CMN taxons				
Mycobacterium murale sp. nov.	10^6 cfu g^{-1}	Immunoreactive cell wall relatedness to pathogenic species	Water damaged gypsum liner	Vuorio et al. 1999
Dietzia sp	10^5 cfu g^{-1}	Immunoreactive cell wall	Water damaged gypsum liner	Andersson et al. 1998
Williamsia muralis gen. nov. sp. nov.	10^3cfu g^{-1}	Not known	Non damaged gypsum liner	Kämpfer et al. 1999
NON-CMN actinomycetes				
Streptomyces griseus	10^5 cfu g^{-1} (gypsum liner) 10^5 cfu g^{-1} (settled dust)	Production of toxic metabolites	Water damaged gypsum liner settled dust	Andersson et al. 1998
Nocardiopsis alba	10^5 cfu g^{-1}	Not known	Water damaged gypsum liner	Andersson et al. 1998

The site from which the *M. murale* strains were isolated had been repeatedly exposed to floor cleaners over a long period of time. Cleaning solutions commonly used in Finland contained 10 % of toluene. Strain MA-112 was found to grow in mineral medium with no carbon source when toluene was added directly to the medium. After three additions of toluene (in 1 week) the turbidity of culture increased from A_{540} of 0.01 to 0.17. Strain MA-168 failed to grow on toluene within one week.

DISCUSSION

Our results show that *Mycobacteria* were major colonizers of a water damaged gypsum liner. The amount 10^6 cfu g^{-1} found in the water damaged liner is of the same order as the amount of *Mycobacteria* found in peatland soils (Katila et al. 1995). The fact that the CMN taxons found in the children's day care center represented new species may indicate that the occurrence and the

taxonomy of these taxons in indoor environment are not well known. The valid taxonomic descriptions of these species are presented elsewhere (Vuorio et al. 1999, Kämpfer et al. 1999, Andersson et al. 1998).

The described species closest related to *Mycobacterium murale* based on comparison of 16S rDNA sequencing were the pathogenic species *M. abscessus* and the non pathogenic species *M. komossense* and *M. aurum*. The antibiotic sensitivity pattern of *M. murale* was similar to that of non-pathogenic environmental mycobacteria (Vuorio et al. 1999). Physiological comparison showed that *M. murale* was related to *M. aurum*, *M. komossense* and *M. vaccae* (Vuorio et al. 1999).

The highest similarity based on comparison of 16S rDNA full sequences was obtained between *M. murale* (Vuorio et al. 1999) and a toluene degrading *Mycobacterium sp.* strain T 104 found in river sediment in USA (Tay et al. 1998). The almost complete 16S rDNA sequences Y08857 and U62890 differed only by six nucleotides in the variable region, from nucleotide 140 to 260 (*E. coli* numbering). One of *M. murale* strains (MA-112) obtained from indoor materials indeed used toluene as a sole carbon source. Toluene is added (10 % v/v) as a disinfectant in a commonly used house cleaning formulation 'Tolu,' popular in Finnish day care centers. Since several other *Mycobacterium* strains, including representatives of *M. vaccae*, isolated from contaminated environment have been shown to utilize toluene (Burback and Perry, 1993; Tay et al. 1998), the use of disinfectants may favor the colonization of indoor building materials by environmental mycobacteria.

CONCLUSIONS

Mycobacteria closely related to pathogenic species may survive and proliferate in water damaged indoor building materials. Use of toluene based desinfectants may favor colonization of indoor building materials by toluene utilizing mycobacteria.

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CELLULAR AND HUMORAL RESPONSES IN AN ANIMAL MODEL INHALING *PENICILLIUM CHRYSOGENUM* SPORES

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ABSTRACT

Penicillium chrysogenum (Pc) is a potential causative agent of the complaints and symptoms of occupants in buildings experiencing "sick building syndrome". Viable Pc spores were recovered from the lungs of mice 15 minutes and 3 hours through 36 hours after intranasal (IN) inoculation of $1x10^6$ spores, of which 25% were viable. Eighteen percent of the viable spores were deposited in the lungs, however, by 12 h, only $1x10^4$ viable spores were recovered. This suggests that the mucociliary tract had cleared the majority of spores deposited, but four percent ($1x10^4$) of the viable spores were retained in the airways and were probably deposited in the alveolar spaces and remained viable for up to 36 h post-inoculation. Similar acute doses of viable spores induced significant ($P<0.001$) increases in tumor necrosis factor α (TNF-α), while non-viable (NV) Pc spores did not. Repeated doses (3 weeks) of $1x10^4$ viable spores induced significant ($P<0.05$) increases in total serum IgE and bronchioalveolar lavage (BAL) interleukin-4 (IL-4), whereas $1x10^4$ NV spores did not. This suggests that viable Pc spores are capable of inducing allergic responses.

KEY WORDS:

Fungus, spores, *Penicillium*, deposition and retention, allergy, asthma, TNF-α, IL-4, IgE

INTRODUCTION

Numerous studies have attempted to elucidate the cause of sick building syndrome (SBS) (Finnigan et al. 1984; Feder, 1985). While no one cause for the symptoms induced by IAQ problems is likely to exist, the presence of fungi in sick buildings is becoming consistently associated with this problem (Mishra et al. 1992; Miller, 1992).

In a previous study, we presented evidence that demonstrated that *Penicillium* species, especially *P. chrysogenum* (Pc), and *Stachybotrys* species are strongly associated with SBS (Cooley et al. 1998). These studies also supported earlier findings that *Penicillium* species have become an important indoor contaminant (Burge et al. 1989). It has been shown that bronchial challenges with *Penicillium* species spores induced immediate and delayed-type asthma in sensitized subjects (Verhoeff et al. 1987). These allergic reactions appear to result from the inhalation of fungal products, but the mechanism(s) responsible for these phenomena remain unclear. This study was designed to examine 1) the deposition, clearance, and retention of *Penicillium* species

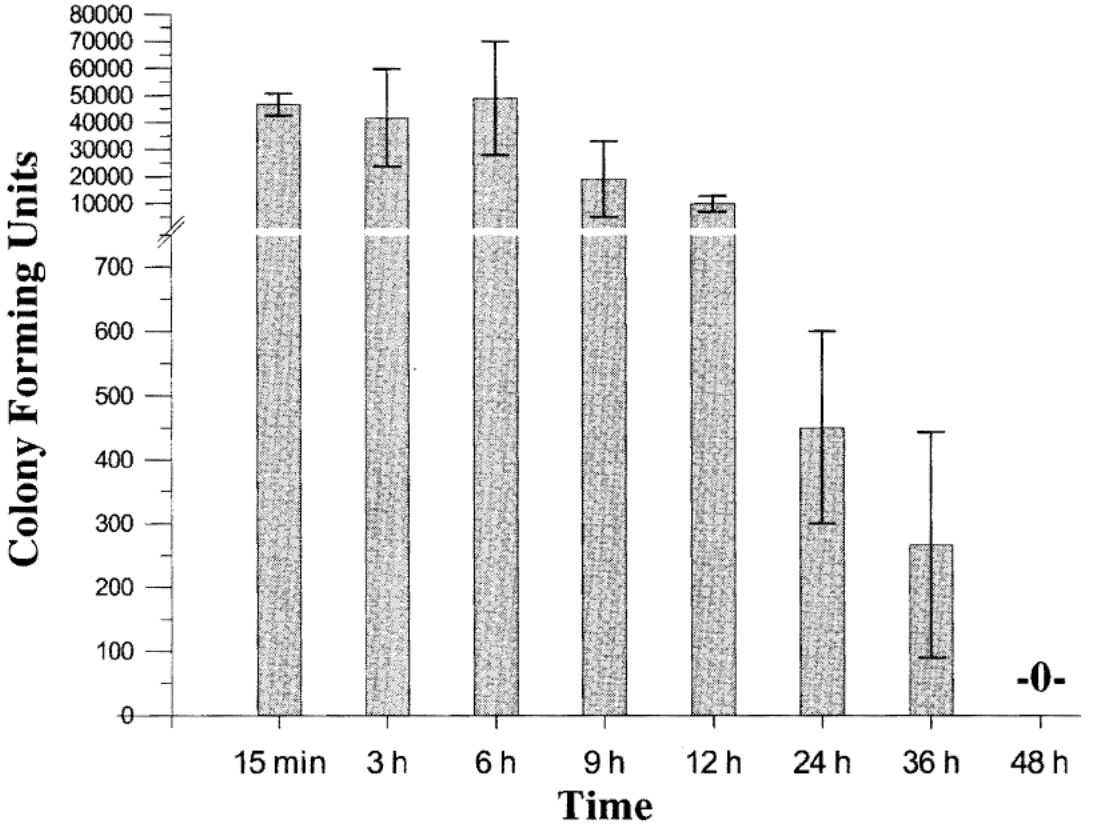
spores introduced intranasally (IN) in a murine model and 2) the consequences of the instillation of Pc spores in the mammalian lung by developing a mouse model.

MATERIALS AND METHODS

Spores.

Pc was isolated from a building that was experiencing SBS and was identified using standard procedures (Frey et al. 1979; Larone, 1993; Rameriza, 1982; Samson and Pitt, 1990). The Pc was grown on Sabouraud's Dextrose Agar (SDA), pH 5.6, for 5 days. The spores were harvested by gentle washing with sterile endotoxin-free phosphate buffered saline (PBS). Preliminary growth studies had shown that only 25% of the spores were viable. Some spores were rendered non-viable (NV) by exposure to methanol. Preliminary experiments had demonstrated that there were no significant differences between this method and other methods used to render the spores non-viable. Spores were counted on a hemocytometer and adjusted to

FIGURE 1: Female C57Bl/6 mice were inoculated IN with $1x10^6$ Pc spores (viability 26%) resulting in a dose of $2.6x10^5$ CFU. At various time-periods after the acute inoculation, the mice were euthanized, the lungs and tracheas aseptically removed, homogenized and serial dilutions plated on SDA plates to determine percent spore viability. Each time-period had a minimum of six mice. The -0- indicates no viable spores were recovered. The error bars represent the Standard Error of Means (SEM).

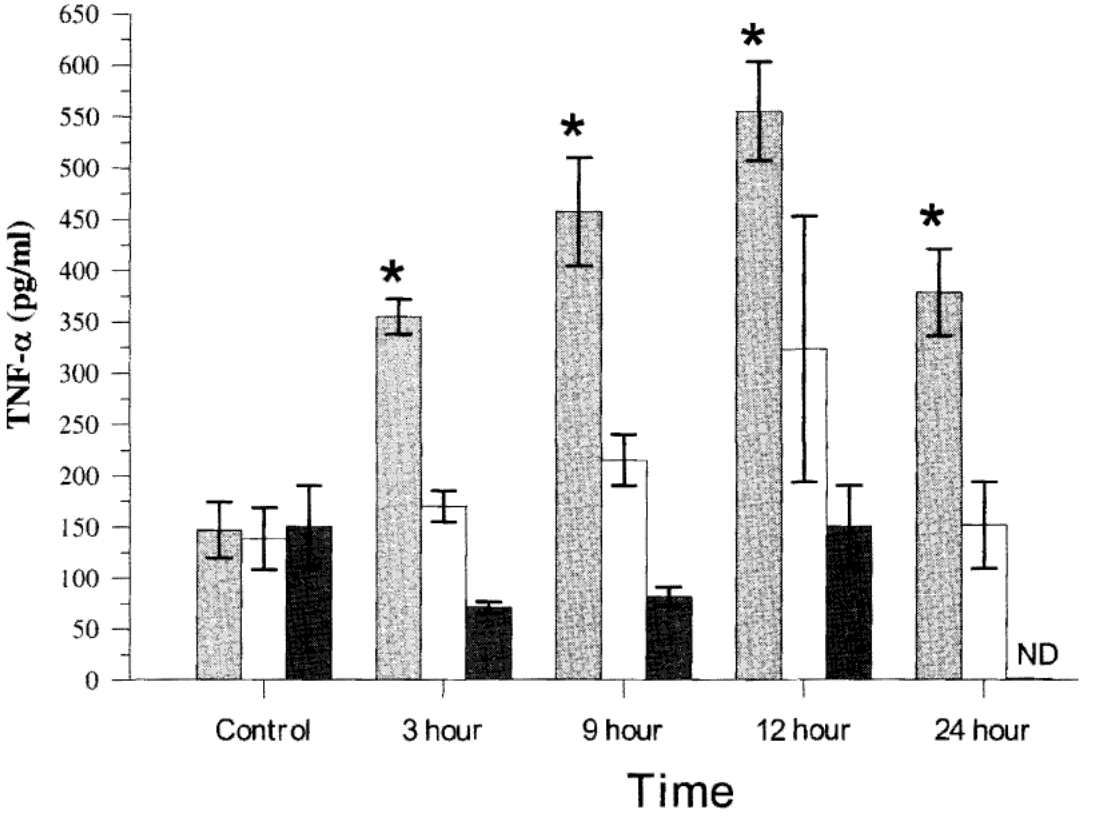


yield $2x10^7$, $2x10^6$, $2x10^5$, and $2x10^3$ singly dispersed spores per ml with average diameter of 3.5μm. Serial dilutions were plated on SDA 5.6 pH plates to determine the percent spore viability.

ANIMALS.

Female C57B1/6 mice (5 to 6 weeks) were maintained in a HEPA filtered room in HEPA filtered metal cages. The mice were lightly anesthetized, placed in an upright position, and inoculated IN with 50μl (25μl per nare) of spores in PBS. The mice were held in this upright position for 2 minutes to allow for complete instillation of the dose. Controls received only PBS.

FIGURE 11: Female C57Bl/6 mice inoculated IN with varying concentrations of Pc spores and at different time-periods, the lungs were lavaged and assayed for TNF-α utilizing a sandwich ELISA specific for murine TNF-α. The light grey bars represent mice that were inoculated IN with $1x10^6$ viable Pc spores; the white bars represent mice that were inoculated IN with $1x10^5$ viable Pc spores; and the dark grey bars represent mice that were inoculated IN with $1x10^6$ NV Pc spores. There were at least 3 mice in each group and time-period. The single asterisk (*) represent $P<0.05$. The ND represents Not Done. The error bars represent the SEM.



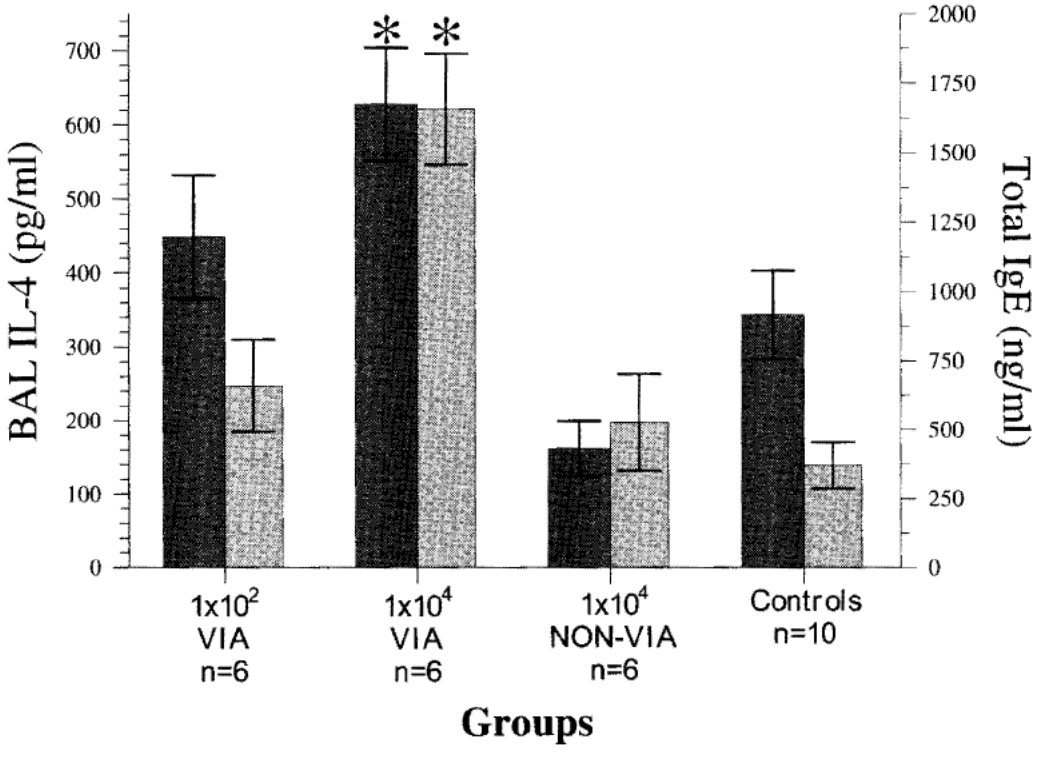
Deposition, clearance and retention experiment.

At various time-periods after acute IN inoculations, the mice were euthanized, and the lungs and tracheas were aseptically removed, placed in 10 ml PBS, and homogenized for 30 seconds. Preliminary experiments had demonstrated no significant loss of spore viability during homogenization for 30 seconds. Serial dilutions were plated on SDA 5.6 pH plates to determine the percent spore viability. There were 3 mice in each group and the experiment was repeated.

Bronchioalveolar lavages and blood samples.

Bronchioalveolar lavages (BAL) were performed The BAL cells were sedimented by centrifugation, stained, and a cell differential count (CDC) performed. The BAL supernatant was filtered and assayed for cytokines. Blood smear slides were stained and CDC performed. Serum was assayed for total IgE.

FIGURE III: Female C57Bl/6 mice were inoculated IN twice per week for three weeks with varying concentrations of Pc spores. Twenty-four hours after the final inoculation, the mice were euthanized, blood processed for serum, and the lungs lavaged. The BAL was assayed for IL-4 (represented by the dark grey bar) utilizing a sandwich ELISA specific for murine IL-4. The serum was assayed for total IgE (represented by the light grey bar) using a sandwich ELISA specific for murine IgE. The single asterisk (*) represents $P<0.05$. The error bars represent the SEM.



Cytokine and immunoglobulin analysis.

The BAL were assayed for tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and interleukin-5 (IL-5), utilizing sandwich ELISAs specific for mouse cytokines (Watson et al. 1993), and total serum IgE using sandwich ELISAs specific for murine immunoglobulins (Coffman and Carty, 1986).

Statistics.

Statistical analysis was performed using analysis of variance (ANOVA) with $\alpha = 0.05$ and $\beta = 0.80$.

RESULTS

Deposition Experiments.

In C57B1/6 mice receiving $1x10^6$ spores (25% viability) IN, spores were recovered from the lungs 15 min and 3 h through 36 h after inoculation. Eighteen percent ($4.7x10^4$) of the viable spores were deposited in the lungs with 4% ($1.0x10^4$) being retained, most likely in the alveolar spaces. Viable spores were recovered up to 36 h post-inoculation (Figure I). The clearance efficiency of the deposited viable spores was 0% for the first 6 h. The clearance efficiency at 9 h was 60%, at 12 h, 79%, at 24 h, 99%, and at 36 h, 99.5%.

TNF-α Production.

C57Bl/6 mice exposed by inhalation to Pc spores demonstrated an increase in TNF-α. There were at least 3 mice in each group and time-period. Mice were inoculated IN with varying concentrations of Pc spores and at different time-periods, the lungs were lavaged to ascertain TNF-α production (Figure II). In this figure, mice received $1x10^6$ spores (26% viability) resulting in a dose of $2.6x10^5$ CFU, $1x10^5$ spores (26% viability) resulting in a $2.6x10^4$ CFU dose, or $1x10^6$ NV spores. The results demonstrate a dose and time-dependent increase in TNF-α production in the viable spore preparations. TNF-α production induced by 10^6 viable spores was significantly different ($P<0.05$) from the controls. There was an increase, although not significant ($P=0.09$), in the number of neutrophils in the BAL cells recovered from the mice that received $1x10^6$ viable spores (data not shown).

3 Week Study.

C57Bl/6 mice exposed by inhalation to viable Pc spores demonstrated an increase in blood eosinophils, BAL IL-4, and total serum IgE. Mice were inoculated IN with $1.0x10^4$ spores (viability 25%) (n=6), $1.0x10^2$ spores (viability 25%) (n=6), $1.0x10^4$ NV spores (n=6), and PBS (controls, n=10) twice per week for 3 weeks (Figure III). The mice receiving 10^4 viable spores demonstrated a significant increase ($P<0.05$) in total serum IgE along with peripheral eosinophilia, while mice receiving 10^2 viable and 10^4 NV spores did not (Figure III). In addition, the BAL from the mice receiving 10^4 viable spores had significant increases ($P<0.05$) in IL-4 (Figure III). In all mice, the BAL cell and IL-5 levels did not differ significantly from the controls.

DISCUSSION AND CONCLUSIONS

There are three methods by which rodents may be inoculated for aerosol deposition. These are 1) natural "physiologic" inhalation, 2) intratracheal instillation, and 3) intranasal instillation (Brain and Valberg, 1979). Each method has its advantages and disadvantages. Natural inhalation requires expensive and complex equipment and chambers along with the technical expertise to generate a constant aerosol. With intratracheal instillation, the same (measured) amount of material can be introduced into each animal. However, this requires deep anesthetization, and repeated anesthetization required for a chronic study could induce comas in the animals. Intranasal instillations require only light anesthetization, but the material must pass through the extensive nasal turbinate network. In addition, the primary objection to intratracheal and intranasal instillation is the high probability that patterns of particle distribution are unlike those resulting from natural inhalation (Brain et al. 1976). However, examination of the immune response to an aerosol deposited into the lungs of an animal depends on the ability of the investigator to introduce known amounts of the material into the lungs. In an earlier study, we demonstrated that occupants in a room in which Pc was growing were constantly exposed to viable Pc spores, with little or no variations in the fungal ecology of the room (McGrath et al. 1998). Since a chronic study using a known amount of spores introduced into lungs was the objective of our lab, we chose to employ intranasal inoculation. Because rodents are obligate nose breathers, the deposition, clearance, and retention of Pc spores in a mouse model were examined.

The retention of a particle is determined by the deposition and clearance of the particle. The output of particles previously deposited in the lungs is called clearance and refers to the processes that physically expel the particles from the lungs. This mechanism includes absorption, sneeze, cough, mucociliary transport, and alveolar clearance mechanisms involving pulmonary macrophages. It should be kept in mind that clearance is often of greater significance than deposition. Therefore, clearance efficiency may be the determining factor for total integrated exposure, and, consequently, the probability of a pathologic or physiologic response, especially when the particles are viable spores.

Figure I demonstrates that approximately 80% of the viable spores were either trapped in the nasal turbinates, swallowed, or expelled. The remaining 20% of the viable spores were deposited distal to the larynx. The clearance efficiency of the viable spores remained close to 0% for the first 6 h, but the PBS carrier liquid was probably absorbed. By 12 h, the clearance efficiency was approximately 80%. This suggests that the remaining viable spores ($1x10^4$) had been deposited and retained in the alveolar spaces, where the primary clearance mechanism involves the pulmonary macrophages. Earlier in vitro studies had demonstrated that pulmonary macrophages had difficulty in phagocytizing viable spores (data not shown). At 36 h, approximately 0.5% of the Pc spores remained viable.

Our data demonstrate that the IN inoculation of viable Pc spores into the lungs of C57B1/6 mice does result in inflammatory and allergic-like reactions. Figure II demonstrates that acute doses of viable Pc spores induced significant ($P<0.05$) increases in TNF-α, while NV spores induced no such inflammatory reaction. TNF-α is a cytokine produced by cells and is involved in the inflammatory cascade (Watson et al. 1993). Figure III shows that a 3 week exposure to

10^4 viable Pc spores induced the production of significant ($P<0.01$) increases in total serum IgE and peripheral eosinophilia. The total IgE increases in this group are supported by the significant increases ($P<0.05$) in the BAL IL-4 from the same spore group. Since IL-4 is required for IgE production (Coffman and Carty, 1986), the production of IL-4 in the lungs suggests that the viable spores were inducing the IgE increase. Since the BAL cells and IL-5 levels from all of the mice did not differ from the controls, this indicates that perhaps the exposure period (3 weeks) was not long enough, especially since we chose not to prime the mice by intraperineal (IP) injection. It is likely that priming animals using IP injections for aerosol sensitization may involve pathways that are not used during a natural sensitization. However, IN priming will probably require longer exposure periods, along with increasing the variability of allergic response in the animals.

Our data suggest that the viability of the Pc spores may be an important factor in the animal's response. The slow clearance of the viable spores suggest that there is ample time for the spores to produce a substance or compound that may contribute to the observed allergic response. One of the most important precipitating factors in asthma is the inhalation of aeroallergen. It is thought that long-term, low level exposure to indoor aeroallergen (e.g., dustmites, molds, pets) may play a role in the generation of asthma and contribute to the chronic and recurring symptomatology (Duff and Platts-Mills, 1992). This study presents more information that the inhalation of fungal spores (specifically viable *Penicillium* species) may play a role in fungal-induced allergies observed in buildings with SBS. This could be by causation or exacerbation.

ACKNOWLEDGEMENTS

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SPORULATION OF THE HYPHOMYCETE STACHYBOTRYS CHARTARUM UNDER THREE LIGHT CONDITIONS

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ABSTRACT

Stachybotrys chartarum is a widespread hyphomycete commonly isolated from a variety of substrates including soil and wood. It can be found growing in building materials, which have become wet before or after construction. The inhalation of *S. chartarum* conidia can cause pneumomycotoxicoses, and a recent study linked *S. chartarum* to the deaths of infants diagnosed with pulmonary hemosiderosis in Cleveland. While *S. chartarum* is frequently isolated, the conditions under which it sporulates are unknown and are important issues in assessing indoor air quality. This study reports on the sporulation of *S. chartarum* under three different artificial light conditions. Two isolates of *S. chartarum* were inoculated onto two different media, MEA and CMA, and exposed to either 24 hour dark, a light/dark cycle, or 24 hour light. After growth initiation, growth rate and degree of sporulation were measured. Results indicate that growth rate and degree of sporulation differ with light conditions and media. On CMA the initial growth rate under 24 hour light was higher than under light/dark and 24 hour dark. Light/dark conditions were more conducive to early sporulation whereas 24 hour dark delayed sporulation. Sporulation did not occur under any light condition on MEA for six days. The data indicate that under favorable growth conditions, *S. chartarum* can sporulate under any light condition. Therefore, *S. chartarum* growing in buildings in dark areas can sporulate for dissemination into the air.

KEY WORDS:

indoor air quality, *Stachybotrys chartarum*, fungal growth, water damaged buildings

INTRODUCTION

Stachybotrys chartarum (Ehrenb. ex Link) Hughes (*S. atra syn.*) is a common, widespread cellulolytic hyphomycete, commonly isolated from a number of different substrates including soil, dead plant materials, wood and hay, particularly under wet conditions and/or high humidity (Domsch et al., 1980). Indoors, *S. chartarum* is frequently isolated from building materials and air samples where water penetration has occurred (Johanning et al., 1993; Hunter et al., 1988; Miller et al., 1988; Bisset, 1987; Croft et al., 1986).

Stachybotrys chartarum produces five stable and persistent macrocyclic trichothecenes, which can inhibit DNA, RNA, and protein synthesis and has long been known to cause a fatal disease in horses. Inhalation of conidia may cause pneumomycotoxicoses, and non-viable conidia, easily dispersed in air under dry conditions, have been shown to have a high concentration of mycotoxins (Sorenson, 1990).

Although *Stachybotrys chartarum* is commonly isolated from a number of ecosystems and is known as a cellulose decomposer, more work needs to be done to elucidate the conditions under which it grows and sporulates. *S. chartarum* has shown a high tolerance to UV irradiation (Durrell and Shields, 1960) and toxin production by *S. chartarum* varied between substrates at different relative humidities (Nikulin et al., 1994). As sporulation is the method of dissemination of *S. chartarum* and inhalation of conidia has been implicated in disease (CDC, 1997; Croft et al., 1986), the conditions under which *S. chartarum* grow and sporulate need to be determined. Several hyphomycetes are known to require light for sporulation; without light, they remain in a vegetative state of growth. This paper presents the results of a study under which *S. chartarum* was exposed to various normal indoor light conditions in which growth rate and degree of sporulation were determined.

METHODS

C.J.K. Wang (1155c) and C. Yang (022098-16) kindly provided *Stachybotrys chartarum* cultures. Isolate 1155c was isolated from the air as a laboratory contaminant, and isolate 022098-16 was isolated from drywall paper. The *S. chartarum* cultures were inoculated onto MEA plates and allowed to grow for 10-15 days, until the new growth was sufficient for transfer. Using a cork borer, 0.5 cm plugs of the new young growth were inoculated onto malt extract agar (MEA) and corn meal agar (CMA) plates. The plates were then incubated under three different light conditions at room temperature: normal daylight/night cycle, complete darkness, and 24 hour light. Five replicates were made for each light exposure.

The colonies were checked every 48 hours for sporulation after inoculation using a dissecting light microscope. Growth rate and the degree of sporulation were recorded. Growth rate was measured incrementally over time. Because the purpose of this experiment was to include the growth rate and degree of sporulation of *Stachybotrys chartarum* under 24 hour dark, it was necessary to inoculate a number of replicated experimental conditions on CMA and MEA. Continuous dark was simulated by wrapping the Petri dishes in aluminum foil. Every 48 hours, one set of the five replicates of each isolate on both media under dark was removed from the aluminum foil and measured. After measurement, these plates were discarded and a new set used for the next measurements.

RESULTS

Growth rate and degree of sporulation were separately analyzed as a function of light conditions and media type. Since the experiment involved the examination of five replicates of two isolates under various light and media conditions, we considered the average degree of sporulation and growth rate for each scenario. Two isolates, or specific cultures of *Stachybotrys char-*

tarion, were observed under the various light and media conditions and different time periods. We used a repeated measures analysis of variance approach (ANOVA), where growth rate and sporulation are measured under three light conditions (light, light/dark, dark) and two media (CMA, MEA) every 48, 96, 144, and 168 hours after inoculation. SAS was used for computations.

Sporulation, although appearing to increase with time, is heavier under light/dark conditions, especially for the 48, 96 and 144 time periods. Under dark conditions, sporulation increases slightly (Figure III). Sporulation on MEA media is practically zero (Figure IV).

A repeated measures ANOVA shows that the degree of sporulation averaged over time is not significantly different among light conditions (P=0.08). However, media is a significant factor (P< 0.001). Testing linear contrasts among light conditions shows that sporulation averaged over time is not different between light and light/dark conditions (P= 0.5), light and dark conditions (P=0.1), but is significantly different among dark and light/dark conditions (P< 0.05).

Growth rate appears to increase with time for both isolates, under each of the light conditions and media. Growth is more robust with more light, but the difference in growth rate among light conditions appears to decrease with time (Figure I). Growth rate is also higher on CMA media than on MEA media with a progressive increase over time (Figure II).

FIGURE I.Growth rate and light conditions.

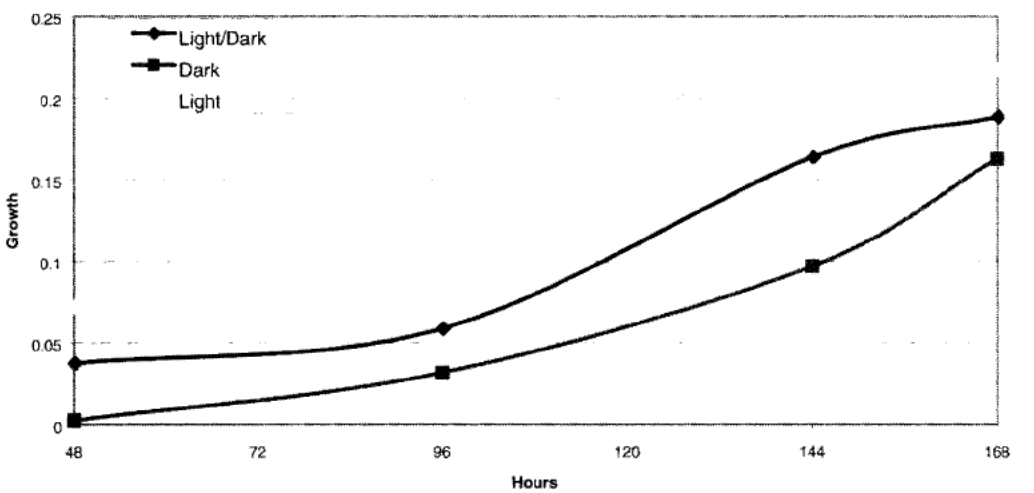


FIGURE II. Growth rate and media.

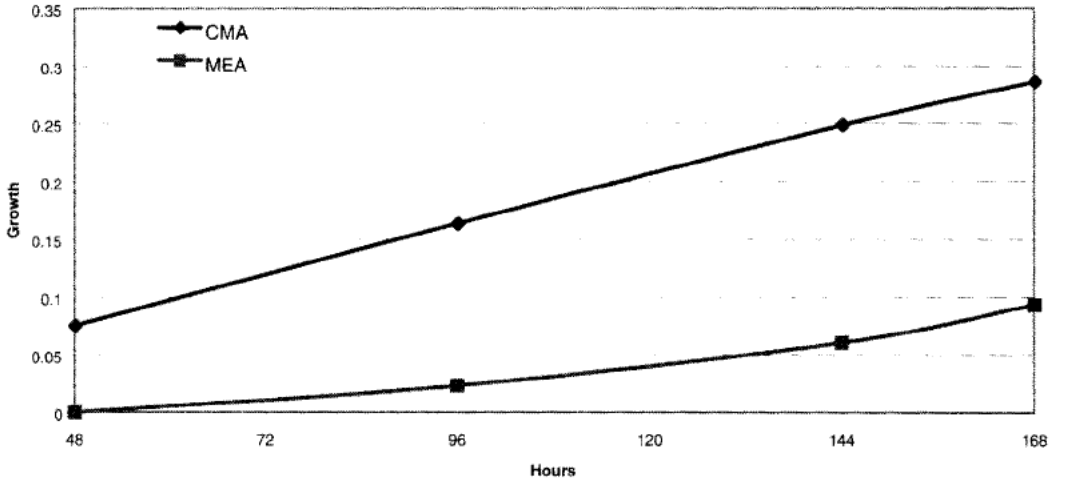


FIGURE III. Degree of sporulation and light conditions.

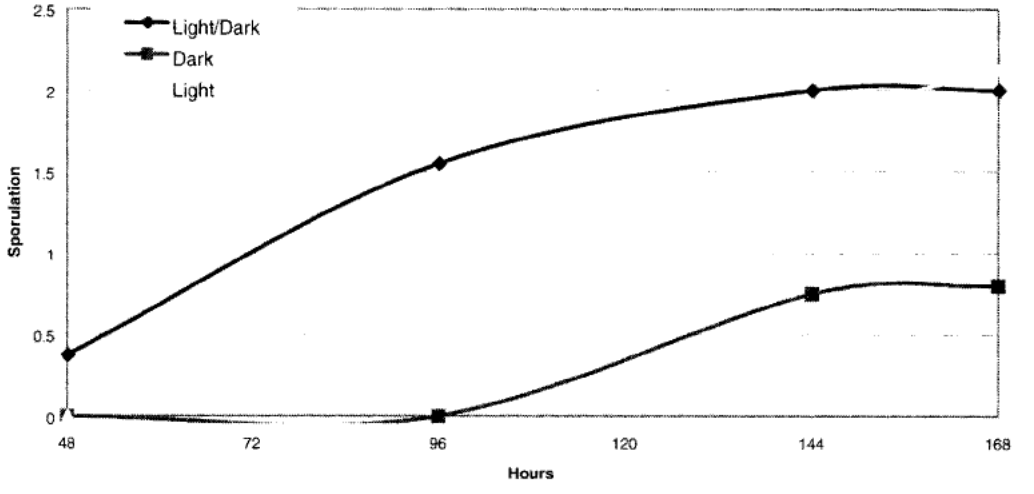
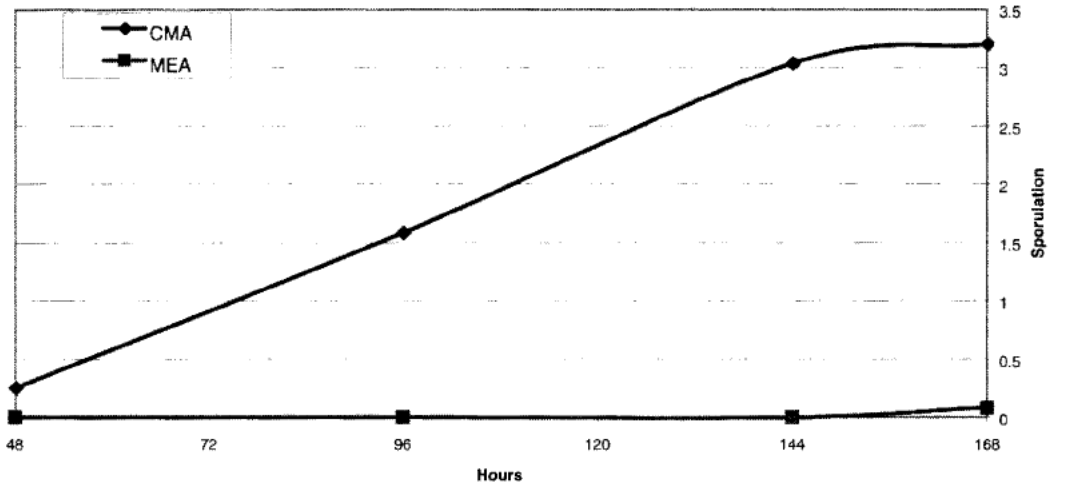


FIGURE IV. Degree of sporulation and media.



Specifically, a repeated measures ANOVA on growth rate shows the following results: growth rates averaged over time differ by light conditions ($P < 0.01$) and media ($P < 0.001$). Specifically, growth rates averaged over time are significantly different between light and light/dark conditions ($P < 0.05$), light and dark conditions ($P < 0.001$), but not among dark and light/dark conditions ($P = 0.06$). This is rather interesting, as the exact opposite patterns were observed for degree of sporulation. This may be due to the effect of light that encourages growth, but sporulation may actually be heavier under light/dark rather than light conditions.

The results indicate that sporulation of *Stachybotrys chartarum*, although delayed, can occur in the dark. *S. chartarum*, when exposed to 24-hour light and light/dark conditions on CMA, sporulated soon after new growth was observed. In general, sporulation was slightly heavier when exposed to light/dark conditions. Sporulation of *S. chartarum* on CMA exposed to 24 hour dark occurred after 144 hours (4 days). While time of sporulation did not differ between the two isolates, isolate 022098-16 (the isolate from drywall paper) was slightly heavier than isolate 1155c.

DISCUSSION

The growth rate of *Stachybotrys chartarum* differs between media and light conditions. Growth rate of both isolates of *S. chartarum* was much lower on MEA than on CMA. This confirms that the growth and spread of *S. chartarum* is dependent on nutrient source. CMA is a richer media containing cellulose, which generally stimulates fungal growth over less nutrient rich media, such as MEA.

Initiation of growth was first observed under light conditions on CMA, and growth rate of both *Stachybotrys chartarum* isolates was generally higher under light conditions. Growth initiation under dark conditions and light/dark cycle was delayed. On MEA, growth initiation was delayed under dark conditions and the growth rate was higher under 24 hour light conditions. In buildings, this could limit the spread of *S. chartarum* under poorer light conditions. This study did not address the growth rate of *S. chartarum* beyond seven days. However, it would be interesting to determine if the growth rate under total darkness would eventually equal the growth rate under 24 hour light conditions.

Under favorable conditions, *Stachybotrys chartarum* can sporulate in the dark. *S. chartarum* sporulated under all light conditions on the CMA, a medium containing cellulose. On MEA, a less nutrient rich medium, *S. chartarum*, in general did not sporulate in less than six days. Because *S. chartarum* is identified to genus based on colonial features and to species by spore characteristics, this implies that samples should not be inoculated onto MEA media unless they are observed for at least seven days when sporulation begins. Our results indicate that when environmental factors including nutrient source are favorable, *S. chartarum* can sporulate under various light exposures. *S. chartarum* is frequently isolated from wetted cellulosic building materials where there is little or no light penetration (e.g., wall cavities). Dissemination of *S. chartarum* is via the production and air dispersal of abundant conidia (Domsch et al., 1980). As *S. chartarum* sporulates under dark conditions, the dispersal of spores is not limited by this environmental condition.

There is increasing concern over the health effects of bioaerosols in water damaged buildings with fungal growth, including growth of *Stachybotrys chartarum*. Recommended procedures for the removal of *S. chartarum* from mold-contaminated buildings have ranged from the removal of building materials supporting its growth to total destruction of the building. Understanding how it grows and sporulates in water damaged buildings is essential in assessing the potential impact on occupant health and safety. *S. chartarum* growing under the varying normal indoor light conditions can sporulate for dissemination.

Air movement is needed to disseminate spores to the breathing zone of a building occupant. Dissemination of spores could depend on a relative difference in pressure between wall cavities and occupied spaces, renovation activities, occupant activities, or destructive testing when growth and sporulation of *S. chartarum* is within wall cavities. If there is no means of dissemination, sporulation alone cannot degrade indoor air quality or impact human health through inhalation. While the risk to humans is still under investigation, spores of *S. chartarum* are known to contain potentially potent mycotoxins, and the spores and hyphal fragments can be allergenic in susceptible hosts. Thus, any environmental condition, such as light, that affects growth and sporulation should be considered when conducting indoor air quality evaluations.

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MYCOTOXIN SPECTRA AS A BIOCHEMICAL PARAMETER FOR OCCUPATIONAL AND ENVIRONMENTAL FUNGUS EXPOSURE

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ABSTRACT

Mycotoxins are metabolites formed by molds in foodstuffs, fodder and organic waste materials. All molds produce specific mycotoxins and species can be characterized by their mycotoxin spectra. We have established a method for mycotoxin extraction from defined cultures. HPLC separation with diode array detection or iontrap mass spectrometry and comparison to an authentic standards library was used to screen seven *Aspergillus* and *Penicillium* species collected at waste treatment plants for their major mycotoxins. The human hepatocarcinogen sterigmatocystin was detected in *Aspergillus versicolor* and *Aspergillus nidulans*. Verruculogen, a potent tremorgen, and fumagillin, formerly used as a cytostatic drug, are mycotoxins found in *Aspergillus fumigatus*. *Penicillium crustosum* and *Penicillium brevicompactum* each produced the tremorgens roquefortine C and penitrem A. The mycotoxin standards library can be used to identify specific mold species in ambient air samples from environmental and occupational investigations.

KEY WORDS

Mycotoxin spectra, mold exposure, biochemical parameter, growth conditions, *Aspergillus*, *Penicillium*, carcinogen, tremorgen

INTRODUCTION

Mycotoxins are fungal metabolites produced in foodstuffs, fodder and organic waste materials. They are known to cause severe diseases, including cancer, upon acute and chronic gastrointestinal exposure. Little data are available on inhalation exposure to these toxins. An increasing number of workers in the waste management industries is exposed to mycotoxin producing *Aspergillus*, *Fusarium* and *Penicillium* species as well as to actinomycetes, which also synthesize secondary metabolites of high toxicity, e. g. dactinomycin.

All fungi produce mycotoxins, and species can be characterized by their mycotoxin spectra. These spectra can serve as an additional approach to measure fungal presence qualitatively and quantitatively. Since determination of cfu reflects only the cultural portion of the fungi, the total organic material from fungi is generally underestimated (Karlsson and Malmberg, 1989).

This dead material may also contain mycotoxins and be of immunopathogenic relevance, causing toxic and allergic effects.

Therefore, we have established a method for mycotoxin extraction from defined cultures of the most abundant mold species collected at German waste treatment plants (Table I). In this study *A. sydowi* was selected to represent the "average" background concentration of molds (100 - 1000 cfu / m^3). *Streptomyces thermovulgaris, Saccharomonospora viridis, Saccharopolyspora rectivirgula* and *Saccharopolyspora hirsuta* were chosen to exemplify actinomycetes.

TABLE I. Most abundant mold species detected in German waste treatment plants (Goettlich et al., 1994; Kutzner and Kempf, 1994).

species	**colony forming units [10^3 cfu / m^3 air]**
Aspergillus fumigatus	9,000
Aspergillus versicolor	7,000
Aspergillus nidulans	3,000
Aspergillus niger	3,000
Aspergillus sydowi	0.1
Penicillium crustosum	600
Penicillium brevicompactum	500
actinomycetes	230

MATERIAL AND METHODS

Mycotoxin spectra depend on the growth conditions of the molds. To evaluate this influence, the selected *Aspergilli* and *Penicillii* were grown on a malt extract agar described by Gams (1987) and a YES-agar according to Frisvad and Filtenborg (1989). Actinomycetes were raised on a caseine peptone - soya powder peptone agar (Difco Laboratories, Detroit, MI, U.S.A.). The fungi were cultivated for 14 days at 25°C; the actinomycetes were grown for two weeks at 37°C or 50°C.

The mycels were gently removed from the plates and washed into round-bottom flasks with methanol. Two volumes of dichloromethane and 0.1 ml formic acid were added to one volume of methanol and the extracts sonicated for twenty minutes. The filtered extracts were taken to dryness under nitrogen and redissolved in 2 ml of methanol prior to HPLC analysis.

50 - 100 µl of each extract was chromatographed at room temperature according to a method outlined by Frisvad (1987). The UV-spectra obtained with diode array detection were compared to 14 mycotoxin references (Sigma Chemical Co., St. Louis, MO, U.S.A.), analyzed under the same chromatographic conditions. To verify the identity of the peak in the original extract, defined amounts of reference substances were added to the extract. Coelution of the reference with the peak under question allowed positive identification of the compound.

In addition, the identified mycotoxins were purified from aliquots of the original extracts and subjected to iontrap mass spectrometry analysis.

RESULTS

Sterigmatocystin was detected in the extracts of *A. versicolor* and *A. nidulans*. The mycotoxins verruculogen and fumagillin were identified as metabolites produced by *A. fumigatus* (Figure I). No metabolic products formed by *A. niger* and *A. sydowi* could be correlated to any mycotoxin of the reference library of 14 substances. Different culture media had no influence on the mycotoxin spectra of the investigated *Aspergillus* species.

Roquefortine C and penitrem A were detected in *P. crustosum* and *P. brevicompactum* grown on YES-Agar (Figure I). The identity of roquefortine C was confirmed by iontrap mass spectrometry in addition to the HPLC-diode array analysis. Both mycotoxins were not produced by *P. crustosum* on the malt extract medium, while *P. brevicompactum* formed only roquefortine C.

Unfortunately, the HPLC-diode array analysis method described above could not be applied successfully to the analysis of the actinomycetes extracts. These extracts seem not to contain any UV-detectable compounds.

DISCUSSION

We have established a HPLC-diode array method to screen pure cultures of molds collected at waste treatment plants for their major metabolites to obtain species specific mycotoxin spectra.

The specificity of the approach is demonstrated in our study: The production of all other mycotoxins on YES-agar for the selected species was consistent with a previous report (Frisvad and Filtenborg, 1989), whereas the formation of roquefortine C and penitrem A and the absence of mycophenolic acid in the YES-agar cultures of *P. brevicompactum* was not. Our *P. brevicompactum* isolate may therefore either be an unusual strain or the microbiological species determination was false. This is currently being reevaluated. Thus, chemotaxonomic information obtained from the mycotoxin spectra may be used to confirm or critically review the microbiological data obtained from the cfu-determination.

Interestingly, mycotoxin spectra of the investigated *Aspergilli* were not influenced by the growth conditions in contrast to the mycotoxin spectra demonstrated with the *Penicillii*. With regard to the varying growth substrates in the waste treatment units, one can conclude that mycotoxin spectra indicating the presence of certain *Aspergillus* species may be a very reliable biochemical parameter.

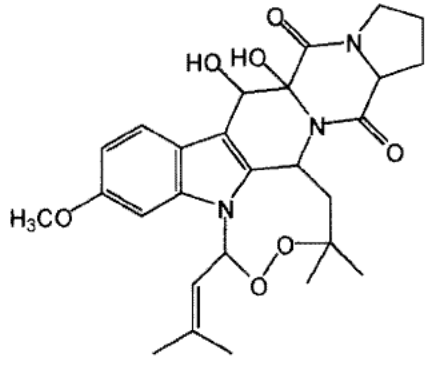
Finally, the compounds identified so far from the cultures are all highly potent carcinogens or tremorgens (Figure I; Hayes, 1980). Their possible contribution to the organic dust toxic syndrome (ODTS) needs to be further evaluated.

CONCLUSIONS

We have established a HPLC-diode array method to be used for the determination of mycotoxin spectra of molds as a biochemical parameter. The specificity of this approach was shown clearly in extracts of pure cultures. The sensitivity of the method will be optimized in order to

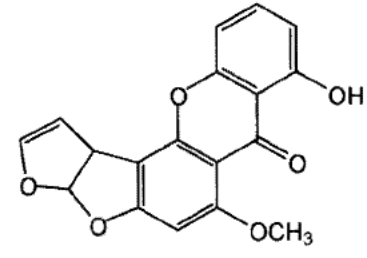
FIGURE I. Structures of mycotoxins detected in extracts of fungus cultures.

A. fumigatus



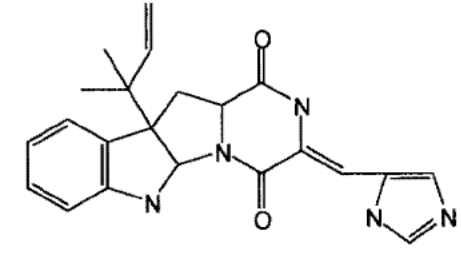
Verruculogen
(tremorgen)

A. versicolor
A. nidulans

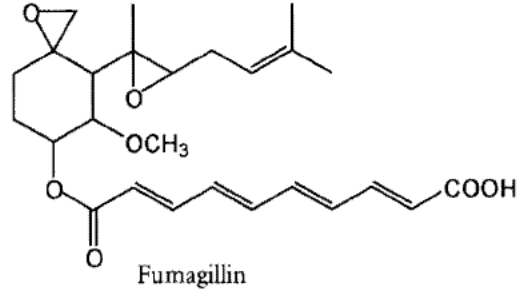


Sterigmatocystin
(Human carcinogen)

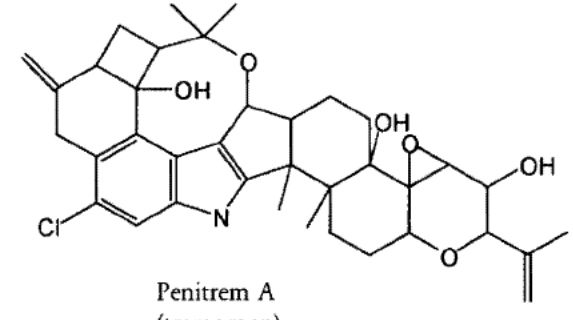
P. crustosum
P. brevicompactum



Roquefortine C
(tremorgen)



Fumagillin
(cytostatic drug)



Penitrem A
(tremorgen)

use mycotoxin spectra as occupational and environmental exposure markers in addition to the standard culture methods.

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PRODUCTION OF MYCOTOXINS ON WATER DAMAGED BUILDING MATERIALS

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ABSTRACT

The ability to produce mycotoxins during growth on inoculated building materials was investigated for *Penicillium chrysogenum, P. polonicum, P. brevicompactum, Chaetomium* spp., *Aspergillus ustus, A. niger, Ulocladium* spp., and *Alternaria* spp. Both isolates of *P. polonicum* produced 3-methoxy-viridicatin, verrucosidin, and verrucofortine. Of five isolates of *P. brevicompactum* two produced mycophenolic acid. *C. globosum* produced the toxic chaetoglobosins A and C. No toxin production was detected from any of the five isolates of *P. chrysogenum*. Alternariol and alternariol monomethyl ether were detected from all five isolates of *Alternaria arborescens*-group and *A. tenuissima*-group. No known mycotoxins were detected from *Ulocladium* spp., and *A. ustus. A. niger* produced several naphtho-γ-pyrones and tetra cyclic compounds. All investigated species, especially *A. ustus* and *A. niger* produced many unknown secondary metabolites.

Analyses of naturally infested materials showed that *Aspergillus versicolor* produced sterigmatocystin and 5-methoxysterigmatocystin *C. globosum* produced the chaetoglobosins A and C, and *P. chrysogenum* produced the non toxic meleagrin.

KEY WORDS:

Mycotoxin, chaetoglobosin, *Chaetomium globosum, Aspergillus, Penicillium, Ulocladium, Alternaria,* chaetoglobosin, alternariol, sterigmatocystin, Indoor, building.

INTRODUCTION

The correlation between dampness, mould growth, and symptoms such as allergy and other respiratory problems, fatigue, irritation of skin, headache, and mucous membrane symptoms e.g. itching eyes and stuffy nose, has been demonstrated during the last 5-10 years (Flannigan et al., 1991; Gravesen, 1994; Husman, 1996; Reynolds et al., 1990; Lacey, 1991; Dales et al, 1997).

Except for specific fungal allergies the causal agents for the symptoms mentioned above have not been found, but at least four fundamental different types of fungal products could be involved:

- PROTEINS found on the surface of the fungal spore, giving the well known allergic reactions (Flannigan et al., 1991; Gravesen et al., 1994; Lacey, 1991).
- (1→3)-β-D-GLUCANS triggering inflammative reactions very similarly to symptoms observed on exposure to endotoxin from gram negative bacteria (Rylander & Fogelmark, 1994; Rylander, 1997).
- VOLATILE ORGANIC COMPOUNDS (MVOCs) released from the fungi during growth, probably triggering inflammative reactions in respiratory passages. It has been shown that MVOCs from *Trichoderma viride* can induce a non IgE mediated histamine release in human mucosal mast cells from the airways in vitro (Larsen et al., 1998).
- MYCOTOXINS including peptides, released from inhaled fungal spores. For example it has been established that macrocyclic trichothecenes produced by *Stachybotrys chartarum* (synonym: *S. atra*) can induce mycotoxicosis in people living or working in buildings infested with this fungus (Etzel et al., 1998; Koscick et al., 1997; Croft et al., 1986; Johanning et al., 1996).

Many of the fungi found on contaminated building materials are well known mycotoxin producers usually demonstrated after growth on foodstuff or laboratory substrates (Larsen & Frisvad, 1994; Hendry & Cole, 1993). Table I shows the mycotoxin-producing ability of the different species used for the experiments described in this study. The list is based on many years of research in fungal chemotaxonomy at Department of Biotechnology (Frisvad & Filtenborg, 1983; Frisvad & Thrane, 1987; 1993; Frisvad, 1998).

Since the production of mycotoxins depends on the substrate, a_W, temperature and other environmental factors (Nielsen et al., 1998b; Frisvad, 1998), determination of the mycotoxins actually produced in water damaged buildings is best done by analyzing contaminated building materials. This has been investigated for: *S. chartarum* producing several macro-cyclic trichothecenes (Croft et al., 1986; Nikulin et al., 1994; Johanning et al., 1996, Nielsen et al., 1998a; 1998b); *A. versicolor* producing sterigmatocystin and 5-methoxysterigmatocystin (Nielsen et al., 1998b); *Trichoderma* spp. from which not any mycotoxins were detected (Nielsen et al., 1998b) and *Alternaria alternata* producing alternariol and alternariol monomethyl ether (Ren et al., 1998).

The scope of this work was to detect the de facto production of mycotoxins by common fungi growing on building materials. This has been done during analyses of artificially infested building materials and material samples from a building recently infested.

TABLE I. Mycotoxin production reported from fungi found in water-damaged buildings[1]

Genus	Species	Secondary metabolites and toxins[2]
Alternaria alter	*tenuissima*	**Alternariol*, alternariol mono ethyl ether*, tenuazonic acid,* toxins I***
Alternaria	*infectoria*	No known metabolites[3]
Alternaria alter	*alternata*	**Alternariol*, alternariol mono ethyl ether*, tenuazonic acid,* toxins I***
Alternaria	sp.	**Altenuene*, alternariol*, iso-altenuene, alternariol mono ethyl ether*, tenuazonic acid,* altertoxins I* & II**, dihydro tentoxin, alterlosin II, tentoxin*
Aspergillus	*niger*	**Naphtho-t-pyrones, Malformins A*, B*, C***, tetra-cyclic com pounds, aspergilin, asnipyrone, asperrubrol, asperenones, aurasperones, 4-hyroxymandelic acid, orlandin*, nigrazines, orobols, pyrophen, flavinines, tubingensin A and B, nigragillin*, kotanin*, Ochratoxin A*[4]
Aspergillus	*ustus*	**Austamide*, austdiol*** and **austocystins A* & B***
Chaetomium	*globosum*	**Chaetoglobosins A*, B** and **C***
Penicillium	*expansum*	**Chaetoglobosins A*** and **C*, communosins A*** and **B***, expan solide*, **roquefortine C*, citrinin*, patulin***
Penicillium	*chrysogenum*	**Roquefortine C***, meleagrin*, chrysogin*, penicillin G*, ω-hydroxyemodine, 2-pyrovoylaminobenzamide*
Penicillium	*brevicompactum*	Botryodiploidin, **Mycophenolic acid***, asperphenamate, bre vianamides A*, B* and F*, brevigellide, drimene X, mycochromenic acid, pebrolides, silvatins, metabolites O[5] and F[5], Raistrick phenoles
Penicillium	*polonicum*	3methoxyviridicatin*, nephrotoxic glycopeptides, **verrucosidin*, normeyhylverrucosidin*, penicillic acid***, verrucofortine*, anacine*, puberuline*, cyclopenin, cyclopetin, viridicatol, dehydro cyclopeptin
Ulocladium	*chartarum*	No known

[1]Samson et al., 1995; Frisvad & Filtenborg, 1983; Frisvad & Thrane, 1987; 1993. *Reference standard at IBT. [2]Toxic metabolites in bold. [3]Andersen & Thrane, 1996. [4]Probably only produced by extremely few isolates. [5] molecular structure unknown.

MATERIALS AND METHODS

NATURAL SAMPLES.

In a domestic residence one sampling was performed by collecting fungal material from heavily infested wallpaper onto a 0.45 µm filter by means of a special sampling device attached to a vacuum cleaner (Dreborg et al., 1995). The other samplings took place in four rooms, where infested wallpaper was easily removed with a scalpel and transported to the laboratory for mycological and chemical analyses.

Artificially inoculated materials

Strains isolated from infested building materials in earlier studies were inoculated on new building materials cut into discs 13 cm in diameter and placed in 14 cm Petri discs according to Nielsen et al., 1998. The discs were streak-inoculated once from a pure culture spore suspension containing 10^5-10^7 spores/ml, incubated in a ventilated room at 25°C, and inspected once a week. Autoclaved double distilled water was added as the materials dried out (7-14 days) to maintain a high water activity.

Mycological analyses

Fungi were identified and cultivated as described by Samson et al., 1995 and Simmons, 1992, and held at the IBT Culture Collection, Department of Biotechnology, Technical University of Denmark. *Aspergillus* and *Penicillium* cultures were identified to species level by morphological characters (Samson et al., 1995) and secondary metabolite profiles using HPLC-DAD (Frisvad & Thrane, 1987; Smedsgaard, 1997).

Chemical analyses

Extraction of the building materials was performed using a modified method of Nielsen et al., 1998b, scraping off the fungal material using a scalpel and then extracting the fungal material using methanol. Extracts were analyzed by high performance liquid chromatography on a Hewlett-Packard HP 1100 HPLC equipped with a diode array detector (Smedsgaard, 1997) and by thin layer chromatography using the methods of Frisvad & Filtenborg (1983), and Frisvad & Thrane (1987).

RESULTS AND DISCUSSION

Table II shows analytical results from the domestic residence and Table III the results from the inoculated materials, it should be emphasized that in the HPLC chromatograms from most of the extracts unknown peaks appeared. Some of the peaks might correspond to known metabolites not included in the IBT metabolite collection, some to unknown metabolites and some to artifacts from the building materials, not appearing in the blank extractions of new building materials.

The sample form the vacuum cleaned wall infested with *P. chrysogenum* revealed meleagrin and ergosterol. Small amounts of sterigmatocystin were also present originating from growth of *A. versicolor* (Nielsen et al., 1998b). Several samples infested with *A. versicolor* contained the carcinogenic sterigmatocystins, confirming the results of Nielsen et al, 1998b. Red colored areas infested with non or poorly sporulating *A. versicolor* biomass contained largest quantities of sterigmatocystins.

Chaetomium spp. consistently produced the cychalasines mycotoxins, including chaetoglobosins A & C on both natural and artificially infested samples, neither chaetocin nor chaetomin were detected in any of the extracts.

On the inoculated materials the secondary metabolism of all the *Penicillium* species were weak compared to the agar media, either due to the small amount of biomass present or to the total-

Table II

Analysis of materials in heavily infested domestic residence

Room	Material	Visual appearance	Area (cm^2)	Isolated fungi	Metabolites[1] (μg)
Kitchen	GF WP[3]	Black discoloring, RV	40	*Ulocladium* sp., *P. chrysogenum*	
Kitchen	GF WP	Green spots and red discoloration, RV	40	*P. chrysogenum*	5-ST (1,4), ST (2,1)
Kitchen	GF WP	Dark grey discoloring, RV	40	*P. chrysogenum*	
Hall	GF WP on WP	Dark green spots on WP	40	*P. chrysogenum*	
Hall	GF WP on WP	Dark green spots on WP	40	*P. chrysogenum*, *Absidia* sp.	5-ST (6,9), ST (70)
Hall	GF WP on WP	Black pinpoint colonies on WP	40	*Chaetomium* sp., *P. chrysogenum*	ST (1,1), CA (34),CC (18)
Hall	GF WP on WP	Black spots on WP	40	*P. chrysogenum*, *Ulocladium* sp.	5-ST(1,8), ST (77),CC (27)
Hall	GF WP on WP	Grey-green spots on WP	40	*P. chrysogenum*	ST (2,7)
Hall	GF WP on WP	Dark olive spots on WP	40	*P. chrysogenum*, *A. versicolor*	5-ST (33), ST (48)
Child's room	WP	Green spots and red discoloration, RV	100	*P. chrysogenum*	5-ST (1,0), ST (1,8)
Office	WP	Dark olive spots on WP	200	*P. chrysogenum*	ST (2,2)
Office	WP	Green spots and red discoloration, RV	200	*P. chrysogenum*	5-ST (15), ST (42)
Office	WP	Dark green spots on WP	200	*P. chrysogenum*	
Office	WP	Black big pin point colonies, extensive biomass	100	*Chaetomium* sp.	
Office	WP	Olive green spots	45	*P. chrysogenum*, *Chaetomium* sp.	
Office	WP	Black big pin point colonies, extensive biomass	25	*Sterilia mycelia*[2]	
Office	WP	Olive green spots	25	*P. chrysogenum*	
Office	WP	Olive green pin points colonies, extensive biomass	100	*P. chrysogenum*, *Chaetomium* sp.	CA (2,7)
Office	WP	Black big pin point colonies, extensive biomass	50	*Chaetomium* sp., *P. chrysogenum*	CA (127), CC (9,2)
Office	WP	Black discoloring with white cotton like colonies, RV	50	*P. chrysogenum*, *Chaetomium* sp.	CA (8,4), CC (4,1)
Office	WP	Dark green spots on WP	60	*P. chrysogenum*, *A. versicolor*	5-ST(7,9), ST (7,3)
Office	WP	Dark green areas, vacuum cleaned	5000	*P. chrysogenum*, *Aspergillus versicolor*	5-ST (2,0), ST(8,5), meleagrin

ST is sterigmatocystin, 5-ST is 5-methoxysterigmatocystin, CA is chaetoglobosin A, CC is chaetoglobosin C, WP is wallpaper, GF is glass fiber wall paper,
RV means reverse side of wallpaper. [1]Using HPLC-DAD. [2]Unknown non-sporulating fungus overgrew the Petri dishes.

Table III

Production of secondary metabolites of fungi artificially inoculated on building materials

No.*	Genus	Species	Gypsum board	Wallpapered gypsum board	Chipboard	Wallpapered chipboard
7028	*Alternaria*	*arborescens*-group[4]	ALT, AME	ALT, AME	ND	ND
7787	*Alternaria*	*tenuisssima*-group[4]	No analysis[3]	ALT, AME	ND	ALT, AME
8478	*Alternaria*	*arborescens*-group[4]	No analysis[3]	ALT[1], AME[1]	ND	ALT
8482	*Alternaria*	*tenuisssima*-group[4]	ALT[1], AME[1]	ALT[1], AME[1]	ND	ALT, AME
8542	*Alternaria*	*tenuisssima*-group[4]	No analysis[3]	ALT, AME	No growth	ALT[1], AME[1]
8485	*Alternaria*	*infectoria*-group	No growth	ND	No growth	ND
15952	*Aspergillus*	*niger*	No growth	ND	N-γ-p[2], TCC[2], nigragillin	N-γ-p[2], TCC[2]
16066	*Aspergillus*	*niger*	No growth	No growth	Orlandin, N-γ-p[2]	N-γ-p[2], nigragillin, Orlandin, TCC[2]
14925	*Aspergillus*	*ustus*	No growth	ND	Kotanins	ND
14926	*Aspergillus*	*ustus*	ND	ND	ND	ND
16810	*Aspergillus*	*ustus*	ND	ND	ND	ND
18350	*Aspergillus*	*ustus*	ND	ND	ND	ND
19563	*Aspergillus*	*ustus*	ND	ND	ND	ND
SK25	*Chaetomium*	*globosum*	Not inoculated	CA[1] (28 μg/cm²), CC[1] (6.7 μg/cm²)	Not inoculated	Not inoculated
8824	*Chaetomium*	*globosum*	Not inoculated	CA[1] (33 μg/cm²), CC[1] (4.7 μg/cm²)	Not inoculated	Not inoculated
8826	*Chaetomium*	*globosum*	Not inoculated	CA[1] (49 μg/cm²), CC[1] (7.3 μg/cm²)	Not inoculated	Not inoculated
8826	*Chaetomium*	*globosum*	Not inoculated	CA[1] (40 μg/cm²), CC[1](4.9 μg/cm²)	Not inoculated	Not inoculated
8827	*Chaetomium*	*globosum*	Not inoculated	CA[1] (42 μg/cm²), CC[1] (5.0 μg/cm²)	Not inoculated	Not inoculated
8828	*Chaetomium*	*globosum*	Not inoculated	CA[1] (24 μg/cm²), CC[1] (5.4 μg/cm²)	Not inoculated	Not inoculated
13972	*Penicillium*	*breuicompactum*	No growth	ND	Siz 1, MYC[1], MET O[1], MET	Siz 1, MYC[1], MET O[1] MET F
13981	*Penicillium*	*breuicompactum*	No growth	ND	Siz 1, MET O[1], MET F	Siz 1, MET O[1], MET F
13982	*Penicillium*	*breuicompactum*	ND	MET O	No growth	No analysis[3]
15953	*Penicillium*	*breuicompactum*	ND	MET O	No analysis[3]	ND
16085	*Penicillium*	*breuicompactum*	ND	No growth	ND	No analysis[3]
17655	*Penicillium*	*breuicompactum*	ND	MET O	No analysis[3]	MYC[1], Siz 1, MET O[1], MET F
15904	*Penicillium*	*chrysogenum*	Not inoculated	Chrysogin	ND	Not inoculated
17491	*Penicillium*	*chrysogenum*	Not inoculated	Chrysogin	ND	Not inoculated
17546	*Penicillium*	*chrysogenum*	Not inoculated	Chrysogin	ND	Not inoculated
19497	*Penicillium*	*chrysogenum*	Not inoculated	Chrysogin	ND	Not inoculated
19569	*Penicillium*	*chrysogenum*	Not inoculated	ND	ND	Not inoculated
14910	*Penicillium*	*polonicum*	ND	3-met	3-met[1], VER, VERU	3-met[1], VER, VERU
14921	*Penicillium*	*polonicum*	ND	3-met, VER, VERU	ND	Not analysed[3]
7712	*Ulocladium*	sp.	ND	ND	ND	ND
8822	*Ulocladium*	sp.	ND	ND	ND	ND
9052	*Ulocladium*	sp.	ND	ND	No growth	No analysis[3]
9051	*Ulocladium*	sp.	ND	ND	ND	No analysis[3]

ND means no metabolites detected. CA is Chaetoglobosin A, CC is Chaetoglobosin C, 3-met is 3-methoxyvirdicatin, N-γ-p is naphtho-γ-pyrones, TCC is tetracyclic compounds, VER is verrucosidin, VERU is verrucofortine, MET O is metabolite O, MET F is metabolite F. *Number in IBT culture collection. 1Confirmed using TLC. 2Known group of metabolite with very specific UV-spectra, specific compound not detected. 3Material discarded due to con

ly different nutrient (Frisvad, 1998). Nevertheless it was possible to detect production of 3-methoxy-viridicatin, verrucosidin, and verrucofortine from *P. polonicum*. *P. brevicompactum* produced the mycotoxin mycophenolic acid and the two structurally unknown metabolites, metabolite F and O (Larsen & Frisvad, 1994). Non-toxic chrysogin was the only known secondary metabolite detected from *P. chrysogenum*, which was in contrast to the naturally infested material where meleagrin were detected.

A. niger produced several known secondary metabolites such as orlandin, nigragillin and more than twenty unknown metabolites including several naphtho-γ-pyrones and tetra cyclic compounds. *A. ustus* produced up to forty metabolites, including several kotanins.

From *Alternaria arborescens*-group and *A. tenuissima*-group (often identified as *A. alternata*), the production of alternariol and alternariol monomethyl ether and three to five unknown compounds with similar UV-spectra was detected from all isolates except from the *A. infectori* isolate. These results are totally in accordance with Ren et al., 1998.

No secondary metabolites or mycotoxins were detected from *Ulocladium*.

It should be emphasized that the mycotoxins detected in this study have been obtained on a limited number of materials. As temperature, a_w and material composition vary extremely in buildings, the mycotoxin production might be different when using other materials and other extrinsic factors (Frisvad, 1998).

CONCLUSION

This study and the papers of Croft et al., 1986; Johanning et al., 1996, Nielsen et al., 1998a; 1998b; Ren et al., 1998; Hodgson et al., 1998, show that many of the fungal contaminants encountered in water damaged buildings actually produce mycotoxins during growth on building materials.

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MEMBRANE TOXIC SUBSTANCES IN WATER-DAMAGED CONSTRUCTION MATERIALS AND FUNGAL PURE CULTURES

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ABSTRACT

We showed toxic substances in and isolated toxin producing microbes from water-damaged building materials. The toxins were extracted from the building materials and microbial cultures in methanol and analyzed using boar spermatozoa as test cells. The fungal genera isolated from the toxic materials were identified as representatives of *Stachybotrys chartarum, Aspergillus, Alternaria,* and *Penicillium*. We found that toxin from the building materials and from four fungal isolates paralyzed sperm cell motility and damaged cell membrane at low concentrations (EC_{50} < 10 µg of methanol soluble solids ml^{-1} of extended boar semen). The toxic isolates were identified to *Stachybotrys chartarum*. The toxin from one *Stachybotrys chartarum* strain was partially purified. To our knowledge, this is the first demonstration of membrane damaging toxin and their producer fungi from the building material.

KEY WORDS

health effects, membrane damage, sick building, sperm cell, *Stachybotrys chartarum*, toxicity, water-damage

INTRODUCTION

In Finland 55 % of houses with moisture problems were reported, exposing ca. 400 000 Finns to microbes (Nevalainen et al. 1998). The reports of water-damaged houses are often based on questionnaires and macroscopic observations with little information on the microbiological status of the building.

Stachybotrys chartarum is often found in buildings reported with water-damage (Andersson et al. 1997; Croft et al. 1986; Johanning et al. 1996) and considered an indicator fungus (Hintikka, Nikulin, 1998). *S. chartarum* is known to produce trichothecenes e.g. satratoxins (Hendry, Cole,

1993; Hintikka, Nikulin, 1998; Sorenson et al. 1987). Airborne trichothecene toxicosis was suggested after detection of massive growth of *Stachybotrys* in building material (Croft et al. 1986). Correlation between exposure to its trichothecenes and lung symptoms was shown with large amounts experimentally injected into the airways (Nikulin et al. 1996). The levels at which trichothecenes are produced at least in laboratory cultures have never so far appeared to be sufficient to cause significant toxic effects (Jarvis et al. 1995). *Stachybotrys* linked cytotoxic effects were seen also in the absence of trichothecenes (Hintikka, Nikulin, 1995).

Sick building syndrome (SBS) refers to emergence of nonspecific symptoms to which generally no single factor can be identified as the dominating cause (Mølhave,. 1998; WHO 1983). The complaints of the occupants of moldy houses can be grouped as i) nonspecific irritation, ii) inflammatory irritation, iii) toxic, iv) nonspecific hypersensitivity and v) allergic reactions (Mølhave, 1998; Rao et al. 1996).

Now its widely recognized among researchers that microbes are involved in health problems in water-damaged buildings but the no single factor have been identified as the dominating cause. We searched for bioactive microbial metabolites in buildings with a history of water-damage where the occupants exhibited nonspecific symptoms. We demonstrate toxic compounds in microbially contaminated building materials and in the cultures of *Stachybotrys chartarum* isolated from the building materials.

MATERIALS AND METHODS

Building materials were extracted and pure microbial cultures were isolated as described elsewhere (Andersson MA et al. 1995; Andersson MA et al. 1997) except for toxicity testing bacterial (28ªC, 10 d) and fungal cultures (22ªC, 10 d) were extracted and redissolved after evaporation in methanol.

Boar spermatozoa were used to test the toxicity of extract prepared from building materials and pure cultures as described by Andersson et al. (Andersson MA et al. 1997; Andersson MA et al. 1998a). The toxic agents were purified as described by Andersson et al. (Andersson MA et al.1998b)

The exposed sperm cells were analyzed by transmission electron microscopy (TEM) as described by Andersson et al. (Andersson MA et al. 1995) except that 2.5 % glutaraldehyde was used for fixation. Plasma membrane integrity was assayed as described by Andersson et al. (Andersson MA et al. 1998a) by epifluorescence microscopy. Analysis of K-76, atranones and trichothecenes (satratoxins) was performed as referenced by Jarvis et al. (Jarvis et al. 1995).

Identification of *Stachybotrys chartarum* isolates was carried out by using species specific primers StacR3 and IT51 published by Haugland and Heckman (Haugland, Heckman,. 1998). PCR conditions were as described by these authors except annealing temperature which was increased up to 63ªC, because lower temperatures produced a very faint product with *Memnoniella echinata* DSM 3469 whereas the product of *Stachybotrys chartarum* was still very strong.

The medical examinations included interview (health symptoms and clinical history), clinical status examination, pulmonary function tests, thorax and maxillary sinus radiographs, complete

blood cell counts, skin prick test to common allergens and molds, and sputum tests (concentration of eosinophilic cation protein, ECP) and determination IgG antibodies to molds and other common allergens.

RESULTS

Toxic substances in water-damaged building materials.

Toxicities of 15 materials collected from buildings with a history of water-damage were assayed for using boar spermatozoa as indicator cells. The results are compiled in Table I. At least three materials were toxic with EC50 values of $\leq$ 5 µg of methanol extractable solids ml^{-1} of extended boar semen corresponding to 60 ... 300 mg of the sampled building material. Two of the toxic materials, gypsum board liner and fiber board, originated from private dwellings and one from a hospital ward (wood), all mechanically ventilated.

TABLE I. Toxicity of indoor building materials. The construction materials from water-damaged buildings where the occupants had nonspecific health complaints (cf. Table IV) were extracted in methanol and tested for toxicity using boar spermatozoa as the test cells.

Sample code	Building		Material sampled	EC_{50}*
	Type	Ventilation		(µg DW ml^{-1})
HMR A	Private dwelling	Natural	**Paper**	> 10.0
HMR B	Private dwelling	Mechanical	Gypsum board liner	< 2.5
HMR C	Hospital ward	Mechanical	**Wood**	< 1.9
HMR D	School	Mechanical	**Gypsum board liner**	> 20.0
HMR E	Private dwelling	Natural	**Wall textile**	1 ... 10
HMR F	Private dwelling	Mechanical	Fiber board	< 1.2
HMR G	Hospital ward	Mechanical	Unidentified	> 11.5
HMR I	Private dwelling	Not known	**Paper**	> 10
HMR J-120	Research center office	Natural	**Fiber board**	> 6.5
HMR J-121	Research center office	Natural	Chipboard	> 12
HMR J-132	Research center office	Natural	**Chipboard**	> 32
P 3	School	Natural	**Felt board**	> 10.5
P 6	School	Natural	**Felt board**	> 5.5
K 5	Day care center	Mechanical	**Insulating material**	> 9.0
K 6	Day care center	Mechanical	**Synthetic carpet**	> 5.0
FB1	University office	Mechanical	Mineral wool**	> 50
SSD1	-	-	Sawdust (spruce wood)**	> 10
BSD1	-	-	Sawdust (birch wood)**	> 10

DW = dry weight of methanol soluble solids extracted from the building material;
*EC_{50} was assayed as the end point dilution causing > 50 % change in vitality parameters compared to spermatozoa exposed to diluent only (= reagent blanc) as described by Andersson et al. (Andersson MA et al.1997.).
**Unused or non water-damaged sites

MICROBIOLOGICAL FINDINGS ON THE TOXIC BUILDING MATERIALS.

Three toxic building materials were analyzed for culturable microbes (Table II). The 13 fungal isolates were identified by microscopy as representatives of *Stachybotrys*, *Aspergillus*, *Alternaria*, and *Penicillium* (Table II). The species identification of *Stachybotrys* sp. isolates was based on species specific PCR. The expected, typical PCR-product for *Stachybotrys chartarum* species, of 200 bp was amplified in all four *Stachybotrys* isolates, HMRB-9, HMRB-10, HMRF-3 and HMRF-4. The four toxic isolates thus were *Stachybotrys chartarum*, with EC50 values < 10 μg ml^{-1} (methanol extract) to boar spermatozoa, equivalent to ≤ 250 μg of fungal biomass ml^{-1} (Table II). For the other fungal isolates of the building materials (Table II) the toxicity thresholds were 1/10 to 1/20 of those of the toxic *Stachybotrys* isolates. The samples also contained < 10^3 to ≥ 10^6 cfu of aerobic heterotrophic bacteria g^{-1} building material but none of the tested 14 bacterial cultures was toxic to boar spermatozoa in the motility assay (Table II).

TABLE II. Culturable microorganisms in toxic building materials. The specimens were rehydrated (1 h) as described elsewhere (1) and aerobically cultured up to 14 d at 28°C on TSA plates (Difco, Detroit, Mich.) to obtain viable cell count and to isolate bacterial strains. The fungi were isolated on media described elsewhere (2) and on sterile filter paper moistened with sterile tap water.

	Heterotrophic bacteria		Fungi		
Sample code	**CFU g^{-1}DW of building material**	**Isolates (n) tested for toxicity**	**Fungal isolate tested for toxicity**	**Toxicity EC50* (μg ml^{-1})**	
				Methanol extract DW	**Biomass WW**
HMR B	1.1 x 10^6	9**	*Penicillium sp* HMRB-7	> 64	> 530
			Penicillium sp HMRB-8	> 68	> 480
			Stachybotrys chartarum HMRB-9	2.5	68
			Stachybotrys chartarum HMRB-10	3.2	82
			Penicillium sp HMRC-3	> 72	> 550
HMR C	2.9 x 10^4	3**	*Penicillium sp* HMRC-4	> 63	> 550
			Aspergillus sp HMRC-5	> 35	> 310
			Aspergillus sp HMRC-6	>3.6 <36	> 60
			Alternaria sp HMRC-7	> 37	> 1020
			Penicillium sp HMRF-1	> 39	> 460
			Penicillium sp HMRF-2	> 27	> 380
HMR F	< 10^3	2**	*Stachybotrys chartarum* HMRF-3	2.4	67
			Stachybotrys chartarum HMRF-4	< 2.5	< 69

CFU = colony forming unit

DW = dry weight, WW = wet weight

* EC50 was assayed as the end point dilution causing > 50 % change in vitality parameters compared to spermatozoa exposed to diluent only (= reagent blanc) as described by Andersson et al. (Andersson MA et al. 1997.).

**No toxicity found in any of the isolates. Toxicity threshold ≥ 3 μg DW ml^{-1}.

EFFECTS ON SPERM CELLS OF THE EXTRACTS FROM THE TOXIC BUILDING MATERIAL AND OF *STACHYBOTRYS CHARTARUM* STRAINS.

Figures I, II and III show the responses of sperm cells exposed to extracts from water-damaged fiber board (Table I) and from *Stachybotrys chartarum* strain HMRF-4 (Table II). The extract prepared from fiber board caused ultrastructural changes in sperm cell. Damage was observed in the outer membrane of the spermatozoan tail after exposure to the extract of the fiber board (Figure IB). Similar damage was generated by a toxic fraction from the *Stachybotrys chartarum* strain HMRF-4 (Figure IIB). This fraction was further purified with RP-HPLC to a single peak. This peak caused similar membrane damage to the exposed spermatozoa (Figure IIC) and in addition mitochondrial swelling (Figure IID). Our results showed that extracts prepared from a microbial pure culture caused ultrastructural damages similar to those evoked by an extract prepared directly from the toxic building material.

FIGURE I: Thin section of middle piece of boar spermatozoa exposed to methanol soluble solids of toxic building material, HMR F. A) Spermatozoon exposed to reagent (methanol) only with intact membranes and ordinary size mitochondria. B) Spermatozoa exposed to methanol soluble solids of toxic building material, HMR F, which was purified by sorption to Sep-Pak column. Demolished membranes are visible (arrows). Bars 0.2 μm.

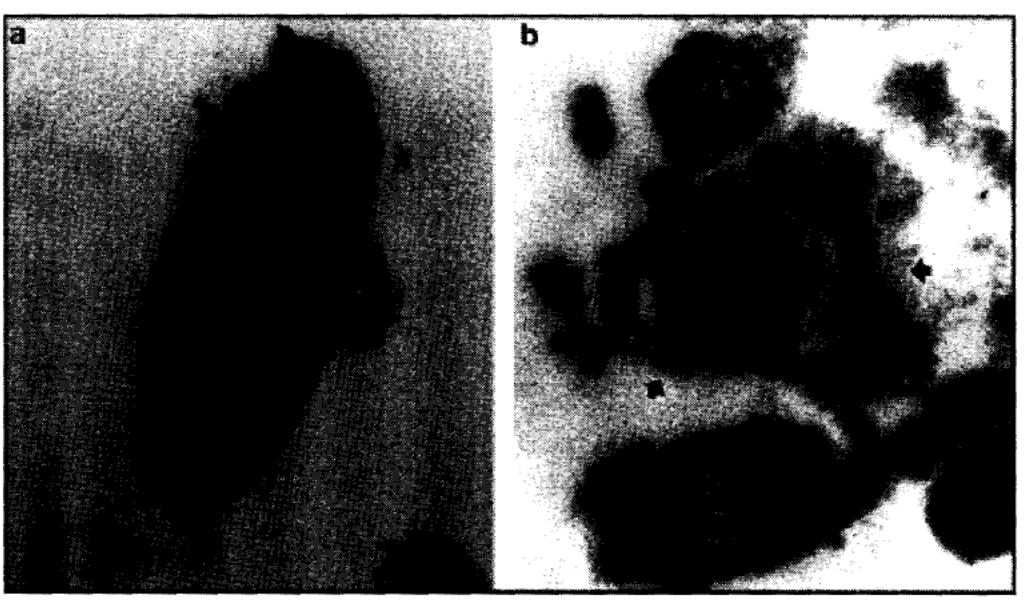


FIGURE II: Thin sections of middle piece of boar spermatozoa exposed (2... 6 d) to fractions of methanolic extract from *Stachybotrys chartarum* strain HMRF-4. A) Spermatozoa exposed to reagent (methanol) only with intact membranes and ordinary size mitochondria. B) Spermatozoa exposed to a fraction purified by sorption to Sep-Pak column and C,D) to a fraction which was obtained from the Sep-Pak eluate by fractionating with RP-HPLC. Demolished membranes (B,C, arrows) and swollen mitochondria (D, arrow) are visible. Bars 0.2 μm.

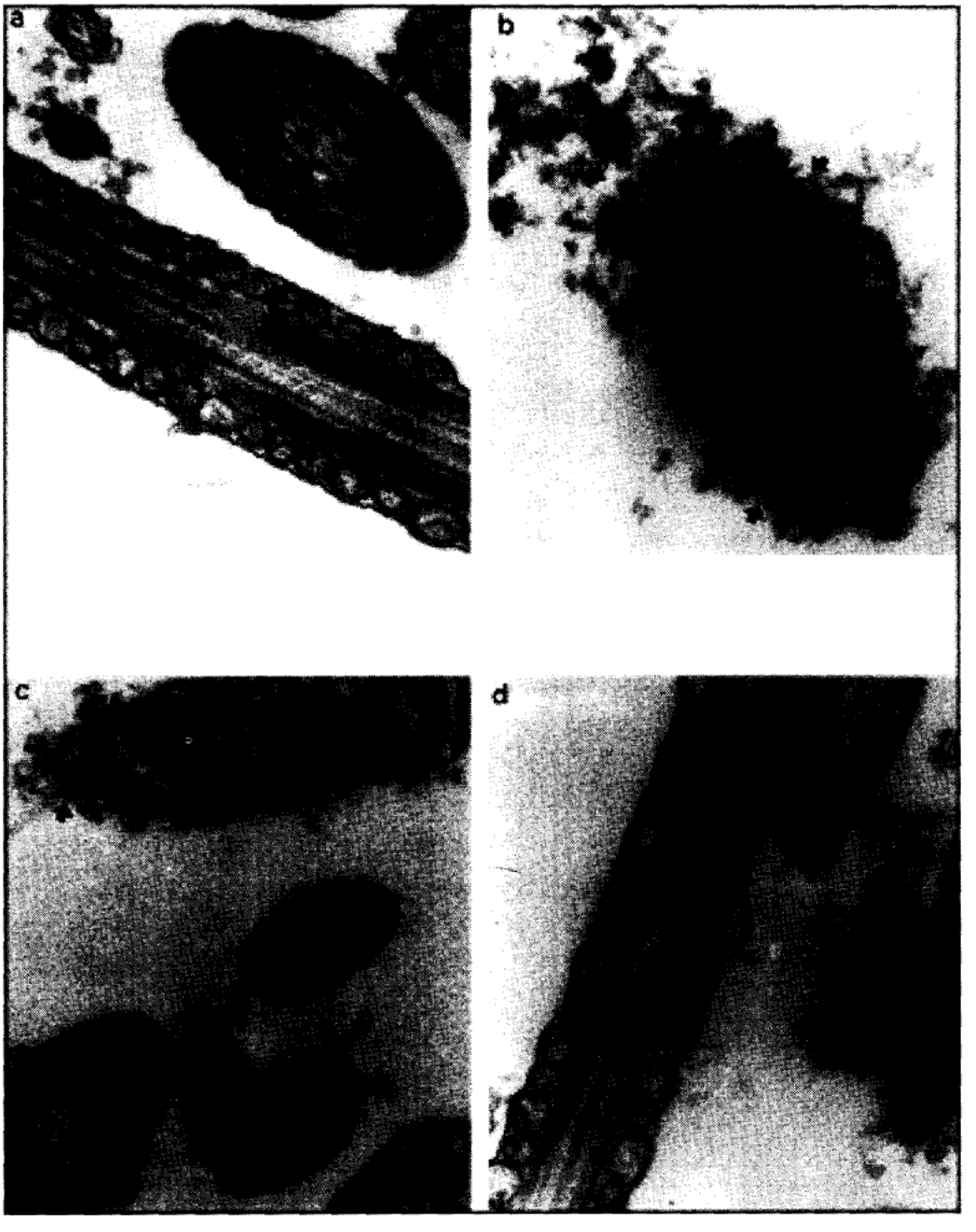
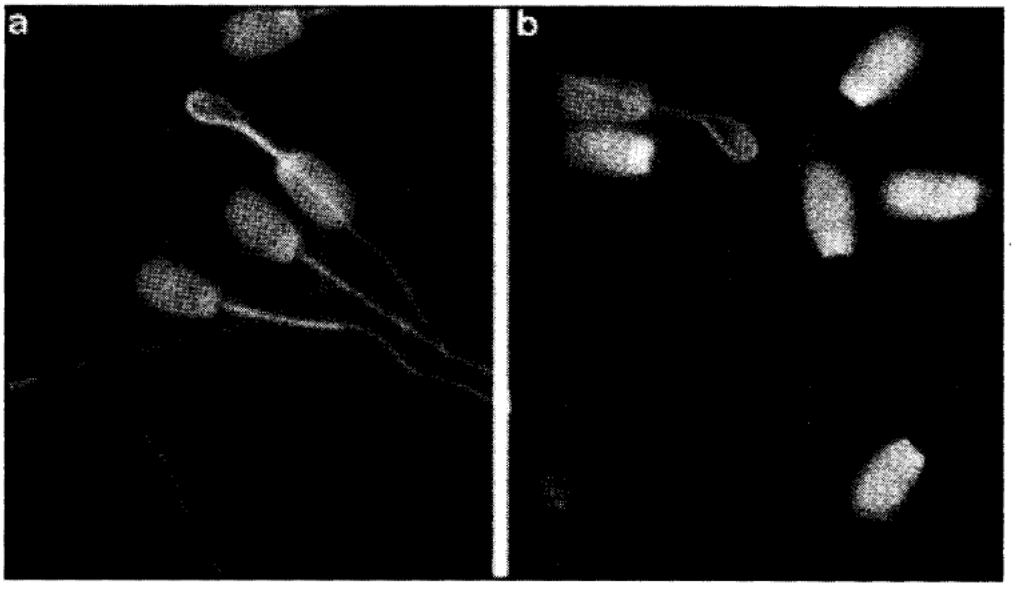


Figure III shows the responses of methanol only and extract from *S. chartarum* strain HMRF-4 exposed spermatozoa to fluorescent vitality stains. The healthy spermatozoa are green (Figure IIIA) and the affected spermatozoa orange due to damage of cell membrane (Figure IIIB). Over 60 % of the spermatozoa were killed (orange) by exposure to 25 μg ml^{-1} methanol soluble solids from *Stachybotrys chartarum* strain HMRF-4. Staining of exposed spermatozoa with the combination of two fluorescent stains, calcein-AM and ethidium homodimer-1, revealed cell membrane damage by extracts *Stachybotrys chartarum* strain HMRF-4 (Figure III).

FIGURE III: Exposed spermatozoa stained with calcein-AM and ethidium homodimer-1. A) Spermatozoa exposed to reagent (methanol) only and B) spermatozoa exposed (3 d) to 25 μg ml^{-1} methanol soluble substances from *Stachybotrys chartarum* HMRF-4. Spermatozoa staining green are undamaged and those staining orange have suffered damage in the cell membrane. Epifluorescence microscope photographs. Magnification 2000x.



Color plates can be viewed on page 671.

The results in Table III show that the agent paralyzing sperm cell motility present in extracts from *Stachybotrys chartarum* HMRF-4 was heat stable (100°C, 20 min. in DMSO) and of small apparent molecular size (< 30,000 g mol^{-1}). The EC50 of this crude extract to sperm cells was lower or similar as that of surfactin and synthetic detergents, SDS, Tween 80 and Triton X-100 (Table III). Purified satratoxin (G and H) inhibited motility at 5 μg ml^{-1} (Table III).

TABLE III: Properties of the toxic agent found in culture of *Stachybotrys chartarum* strain HMRF-4 and reference materials.

Extract or reference material	EC_{50}* (µg DW ml-1)	Inactivated by boiling	MW g mol-1	Membrane damage**
Stachybotrys chartarum				
HMRF- 4 methanol extract	< 2.5	no	< 30 000***	**yes**
Satratoxin (G and H)****	5	ND	ca. 520	ND
Trichothecene T-2****	0.5	ND	466	ND
Sodium dodecyl sulfate (SDS)	1... 10	ND	288	yes
Tween 80	1... 10	ND	ca. 1200	yes
Triton X-100	1... 10	ND	647	yes
Surfactin	5... 6	ND	1036	ND

DW = dry weight, MW = molecular weight
ND, not done
* EC_{50} was assayed as the end point dilution causing > 50 % change in vitality parameters compared to spermatozoa exposed to diluent only (= reagent blanc) as described by Andersson et al. (2).
**Observed by TEM.
***As solution in 50 % (v/v) methanol permeating through 30kD microconcentrator membranes (Amicon Inc., Beverly, Mass).
****Determined by Andersson et al. (Andersson MA,et al. 1997).

Sodium dodecyl sulfate (SDS) and surfactin were obtained from Sigma (St. Louis, Mo.), Triton X-100 was from Boehringer (Mannheim, Germany) and Tween 80 from Fluka (Buchs, Switzerland).

Crude methanolic extracts from *Stachybotrys chartarum* strains HMRB-9, HMRB-10, HMRF-3, and HMRF-4 were similarly toxic to spermatozoa whether the fungi were grown in the absence or the presence of streptomycin sulfate (40 µg ml^{-1}, Sigma, St. Louis, Mo). This indicates that the toxic substances were produced by fungi not by bacteria possibly accompanying the fungal isolates. The *S. chartarum* strains HMRB-9, HMRB-10, HMRF-3 and HMRF-4 all contained significant levels of K-76 and spiro compounds. Atranone-6 (dollabellatriene) was detected in all four. No trichothecenes (eg. satratoxins) or other atranones were detected.

When the *S. chartarum* strain HMRF-4 was repeatedly cultivated on malt extract agar it lost toxicity. The loss was phenotypic rather than genotypic because toxicity was restored after the strain was cultivated on sterile filter paper. This may indicate a connection of the toxin to the cellulose degrading process.

Clinical symptoms of patients.

Twenty persons living or working in buildings, where the water-damaged materials (Table I) were obtained, reported in total 22 different nonspecific symptoms listed in Table IV. The symptoms most frequently reported was irritation of the eyes (15 out of 20) and the nasolaryngeal area (11 out of 20, Table IV). Table IV also shows that 3 of 6 persons exposed to materials HMR B, HMR C and HMR F with shown toxicity suffered from irritation of skin, rhinitis, fatigue and had IgG antibodies against *Stachybotrys*. The other symptoms listed in Table IV seemed to vary individually. The results do not reveal any clear group of symptoms that could be connected to the buildings from which toxins were found and described in this paper.

Table IV: Symptoms, disorders and clinical findings of occupants of the water-damaged buildings described in Table I.

Exposed to* HMR	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**J**	**Total**
Symptoms and illnesses	**with symptoms or disorders / exposed or studied**								
Irritation of skin	1/4	0/1	0/1	1/3	1/5	3/4	0/1	1/1	6/20
Nasal congestion	1/4	1/1	1/1	1/3	2/5	0/4	1/1	1/1	8/20
Irritation of eyes	4/4	0/1	1/1	3/3	4/5	1/4	1/1	0/1	15/20
Dizziness	1/4	0/1	1/1	0/3	0/5	0/4	1/1	1/1	3/20
Nausea	1/4	0/1	1/1	0/3	2/5	0/4	0/1	1/1	5/20
Sinusitis	1/4	1/1	0/1	0/3	1/5	0/4	0/1	0/1	4/20
Cough, hoarseness or sore throat	3/4	0/1	0/1	2/3	4/5	1/4	1/1	0/1	11/20
Headache	1/4	0/1	1/1	1/3	3/5	0/4	0/1	1/1	6/20
Fatigue	1/4	0/1	1/1	2/3	1/5	3/4	1/1	0/1	10/20
Rhinitis	1/4	1/1	0/1	2/3	2/5	3/4	0/1	1/1	9/20
Dyspnoea	0/4	1/1	1/1	2/3	0/5	1/4	1/1	1/1	7/20
Arthralgia	0/4	0/1	0/1	0/3	2/5	0/4	0/1	1/1	3/20
Slight fever	0/4	0/1	0/1	2/3	4/5	0/4	1/1	1/1	8/20
Otitis	0/4	0/1	0/1	0/3	3/5	0/4	0/1	1/1	4/20
Other**	0/4	1/1	0/1	0/3	3/5	0/4	0/1	1/1	5/20
Clinical findings HMR									
Serum IgG:									
Stachybotrys chartarum positive	2/4	1/1	1/1	1/1	1/2	2/4	0/1	1/1	9/17
Aspergillus fumigatus positive	4/4	1/1	1/1	3/3	2/2	1/1	0/1	1/1	13/14
Sputum ECP conc. increased	1/3	0/1	0/1	2/3	1/2	1/1	0/1	0/1	5/13
Lymphocytosis (BAL)	1/2	1/1	ND	1/3	0/1	ND	1/1	1/1	5/9

BAL = bronchoalveolar lavage, ECP = eosinophilic cation protein
ND, not done
*For codes see Table I.
**Included dry throat, chest pain, exacerbated psoriasis, sanguinilent sputum, mucous secretion, prolonged flu.

DISCUSSION

In this paper we report on microbially contaminated, a sick building syndrome related, water-damaged building material from which we isolated toxic microbial cultures. The isolated cultures displayed similar toxicity on cellular level as did the building material. The toxins were partially purified and shown to be cell membrane damaging. To our knowledge, this is the first demonstration of membrane damaging fungal toxins isolated from building material.

The four toxic fungal isolates all were *Stachybotrys chartarum*, based on species specific primers described by Haugland and Heckman (Haugland, Heckman,. 1998). These authors studied 15 *S. chartarum* strains, 12 other *Stachybotrys* species, *Memnoniella echinata* (UAMH 6594), *Memnoniella subsimplex* (ATCC 22699) and found primers StacR3 and IT51 to be specific for *Stachybotrys char-*

tarum. This genus is a known producer of bioactive and/or toxic compounds (Hendry, Cole,. 1993; Sorenson et al. 1987). We noticed that one of the *Stachybotrys chartarum* isolates (HMRF-4)ceased to produce the toxin after repeated cultivation on malt extract medium. Interestingly the toxicity was restored upon cultivation on filter paper. The Canadian and Finnish Guidelines (Anonymous. 1995; Anonymous. 1997) recommend malt extract medium to detect fungi in building materials. Doing so toxicity of *Stachybotrys chartarum* thus may remain unnoticed. A high ratio of carbon to nitrogen in growth medium was observed to give the highest yields of trichothecenes by *Fusarium* sp. (Desjardins et al. 1993). Wheatley et al. (Wheatley et al. 1997) noted that the emission of VOCs and inhibitory effect by *Trichoderma* sp. against the wood decay fungi were greater when cultivated on malt extract agar than on minimal agar. This is opposite of what we noticed with *Stachybotrys chartarum* HMRF-4. One explanation for this could be that *Stachybotrys chartarum* HMRF-4 produced the toxin to repress competing microorganisms when degrading woody or cellulosic (paper) construction materials.

We showed earlier that methanol extracts from water-damaged building materials paralyzed sperm motility and swelled mitochondria at ppb dosage level (Andersson MA et al. 1997). In this paper we show that an agent isolated from *Stachybotrys chartarum* strain HMRF-4 also provoked mitochondrial swelling in addition to demolishing cell membrane of the sperm cells. Known surface active compounds (Table III) paralyzed sperm cell motility and demolished the cell membrane of the spermatozoa similarly as did the extracts from *Stachybotrys chartarum* strain HMRF-4 indicating that one of the toxins produced by *Stachybotrys chartarum* strain HMRF-4 may be surfactant like. The mitochondria swelling potency was observed in purified toxin preparations of *Stachybotrys chartarum* strain HMRF-4 whereas in non-purified preparations the membranolytic activity was prevalent. *Stachybotrys chartarum* strain HMRF-4 may therefore have produced two or more different toxic agents. Concentrations as low as 2...3 $\mu g\ ml^{-1}$ of the cell extract of this isolate paralyzed sperm motility compared to over 5 $\mu g\ ml^{-1}$ of purified satratoxin (G and H) required to inhibit sperm motility. The four toxic *S. chartarum* strains, including HMRF-4 were shown not to produce trichothecenes. These results show that the toxicity of HMRF-4 could not rely on satratoxin.

While airborne toxicoses have been described in heavily by *Stachybotrys chartarum* contaminated building materials (Croft et al. 1986), only low levels of satratoxin have been reported (Andersson MA et al.1997). It is likely that no single toxic agent will explain the myriad of health symptoms observed (Table IV), more likely it is a multifactorial effect.

The toxic agents produced by the *Stachybotrys chartarum* strain may have been accumulating under long periods of time when building materials suffered repeated water-damages. The conidia of *Stachybotrys* sp. are usually produced in slimy heads thus they are aerosolized only when dry or disturbed. While *Stachybotrys chartarum* was found in water-damaged building, IgG antibodies to *S. chartarum* were not statistically connected to health outcomes (Johanning et al.1996). Thus allergic reaction requiring direct contact to the fungal hyphae or conidia, are not necessarily connected to *Stachybotrys* sp. findings in damp houses. The surfactant like toxic agent of *Stachybotrys chartarum* strain HMRF-4 may potentiate the effects of other toxins and contribute to the etiology of health symptoms associated with indoor air also in low doses.

The complaints observed by us, e.g. irritation of eyes, headache, sore throat, running or blocked nose, and fatigue, have also been reported in damp houses where *Stachybotrys chartarum*

(*atra*) was detected (Hintikka, Nikulin, 1998; Johanning et al. 1996; Reijula 1998; Rylander, 1998). The liquid layer of eyes was reported to sorb water-soluble compounds, like surfactants thereby reducing the surface tension of the tear film (Johanning et al. 1996). The membranolytic agent of *Stachybotrys chartarum* (Figure II) may explain the frequent eye symptoms observed in the present study.

CONCLUSION

We show the presence of cell membrane damaging agents, distinct from satratoxin, in water-damaged building materials possibly involved in the etiology of health symptoms associated with indoor air. The toxic agents were detected using sperm cell motility assay in extracts prepared directly from affected building materials. We isolated from the building materials fungal cultures, of which four *Stachybotrys chartarum* strains proved to be capable of producing similarly reactive compounds as those found in the building materials.

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EFFECTS OF *STACHYBOTRYS CHARTARUM* SPORES AND TOXIN ON ALVEOLAR SURFACTANT PHOSPHOLIPID COMPOSITION AND CONCENTRATION IN MICE.

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ABSTRACT

Surfactant is an important phospholipid-rich surface coating material produced by alveolar type II cells. It promotes lung stability by reducing the surface tension of the air-alveolar interface, and serves in alveolar defense. In this study, we evaluated the effects of *Stachybotrys chartarum* conidia and a trichothecene, isosatratoxin-F, on the concentration of phospholipids in alveolar surfactant subtypes in mice. Alveolar surfactant phospholipid content in the total surfactant and the different metabolic subfractions of lung lavage fluid of mice showed some significant changes within 24 h post-exposure, compared to the surfactant from control mice which were either untreated, exposed to saline or *Cladosporium cladosporioides* spores. In both the *S. chartarum*- and the isosatratoxin F-treated mice, exposure significantly increased lysophosphatidylcholine (LP) concentrations in total surfactant. Exposure to *S. chartarum* also resulted in an increased sphingomyelin (SP) and phosphatidylserine (PS) and depressed phosphatidylcholine (PC) content in surfactant. The isosatratoxin F-treated mice also supported depressed phosphatidylinositol (PI) and phosphatidylglycerol (PG) surfactant concentrations. Compared to both of the controls, *S. chartarum*-treated mice had significantly elevated SP in the P10 subfraction, phosphatidylserine (PS) in the P60 and P100 subfractions and significantly elevated PC content in the LB fraction. These mice also showed significant depressed PC content in the P10 subfraction. Isosatratoxin-F-treated mice had significantly elevated PC and PE concentrations in the P10 and P60 subfractions and depressed PE concentrations in the P100 subfraction. In contrast, *C. cladosporioides*-exposed mice showed significant elevations in PE and PC in the P10 subfraction and LB fraction, respectively, and a significant depression in SP concentration in the LB fraction. While the mechanisms of cytotoxicity are unclear, these results reveal that alveolar type II cells are sensitive to exposure to *S. chartarum* conidia and to isosatratoxin F. Sensitivity is manifest by alterations in the normal metabolic processing of alveolar surfactant. In exposed mice, this effect appears to involve significant changes in the content of phospholipids in total and fractionated surfactant from mice. These results further indicate that alveolar type II cells show high sensitivity toward *S. chartarum* conidia and toxins, and support our earlier hypothesis that *S. chartarum* influences both synthesis and secretion of pulmonary surfactant in the lung.

INTRODUCTION

The effects *Stachybotrys chartarum* (*atra*) and some its trichothecene metabolites on respiratory cells such as alveolar macrophages is well understood. However, their effect on another important group of cells in the respiratory system, alveolar type II cells, is not. Alveolar type II cells comprise 60% of the cells of the pulmonary epithelium (Burkitt et al. 1993). These cells synthesize and store major components of pulmonary surfactant (Stratton, 1984), a phospholipid-rich substance that lines the alveolar surface. Surfactant promotes lung stability by reducing the surface tension of the air-alveolar interface (Guyton et al. 1984; Notter, 1984), and it performs important functions in alveolar defense (Curti and Genghini, 1989; Jarstand, 1984; Schurch et al. 1990).

Alveolar surfactant comprises at least four subfractions, P10, P60, P100, and S100 that are thought to represent its different metabolic forms (Oulton et al. 1993). The heaviest subfraction, (P10), is secreted by the alveolar type II cells, and is responsible for the surface-tension-lowering properties of the lipid monolayer on the alveolar surface (Guyton et al. 1984). The other subfractions, P60, P100 and S100, constitute the intermediate and "metabolically-used" forms of surfactant, respectively (Oulton et al. 1993). Inhalation exposure to pollutants such as ozone, nitrogen dioxide, and polyurethane smoke can cause disturbances in the cycling of surfactant once it has been secreted into the alveoli. These can lead to alterations in the quantity, composition, and distribution of its components (Gross, 1991; Higuchi et al. 1992; Oulton et al. 1993, 1994; Scott et al. 1988). In turn, these perturbations may alter alveolar surface tension, interstitial hydrostatic pressure, and capillary fluid entering the interstitium (Rhoades and Pflanzer, 1996). In severe cases, perturbation in surfactant production by pollutants can cause respiratory distress syndrome and death (Rhoades and Pflanzer, 1996).

The main chemical constituent of surfactant is phosphatidylcholine (PC), which represents some 70-80% of the phospholipid, and over 60% of this is disaturated phosphatidylcholine (Haagsman and van Golde, 1991). Most of the remainder of the phospholipid content is derived from PC and distributed among some seven members including, in descending order of occurrence, phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PA), lysophosphatidylcholine (LP) sphingomyelin (SP), phosphatidylethanolamine (PE) and phosphatidylserine (PS). The concentrations of these phospholipids remain in relatively constant proportion in surfactant in healthy individuals (Haagsman and van Golde, 1991).

We have recently shown that alveolar type II cells are sensitive to exposure to *S. chartarum* conidia and to isosatratoxin F (Mason et al. 1998). Sensitivity is manifest by alterations in the normal metabolic processing of alveolar surfactant. In exposed mice, this effect appears to involve a significant increase in newly secreted surfactant and an accumulation of the used surfactant forms. What is unclear from our recent studies is whether there are concurrent changes in the composition of surfactant phospholipids in exposed animals. The objective of this study was to evaluate the effects of *S. chartarum* spores and the *S. chartarum*-derived trichothecene, isosatratoxin F, on surfactant phospholipid composition and concentration, using live juvenile mice.

MATERIALS AND METHODS

Figure I: Schema showing primary steps employed in the experimental protocol for the treatment of animals, and the isolation and analysis of lung surfactant phospholipids.

Intratracheal Instillation Regime

Stachybotrys chartarum (50ul x10^6spores/ml/animal)
Isosatratoxin F (50(l x 10^{-7}M isosatratoxin-F)
Cladosporium cladosporioides (50ul x10^6spores/ml/animal)
Saline (50ul physiological saline/animal)
Untreated Animals

24 h exposure

Lung Tissue recovery and homogenization

Lung Lavage Fluid Recovery

Subfractionation of Surfactant by Differential Centrifugation
10,000g (30 min), 60,000g (60 min), and 100,000g (15 h)

Assay Surfactant Subfractions for Phospholipid Concentration

Separate Phospholipid Constituents Using TLC
Chloroform/Methanol/Water Solvent

Phospholipids Removed from TLC Plates and Digested in Perchloric Acid for Phospholipid Concentration Determinations

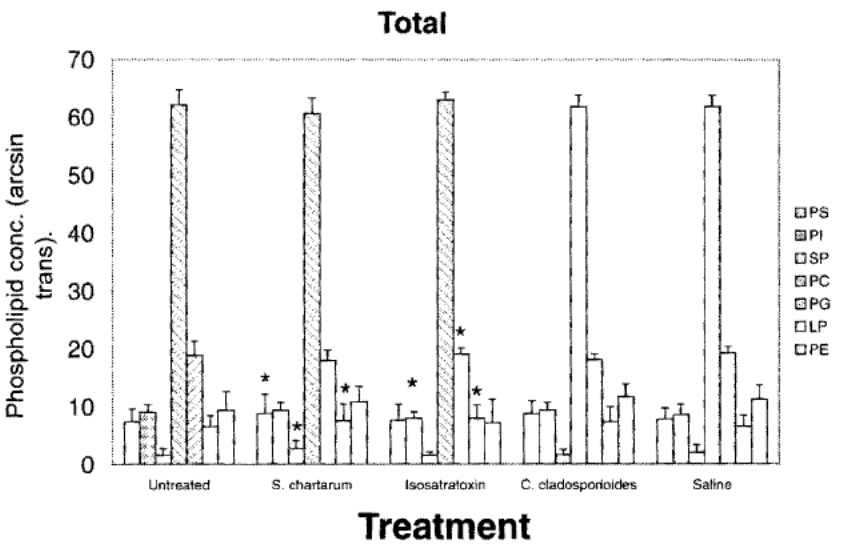
Statistical analyses

RESULTS

Figure II shows that exposure to *S. chartarum* spores and isosatratoxin-F resulted in some significant changes in the phospholipid concentrations in total surfactant recovered from lavage washings from mice. In mice intratracheally exposed to *S. chartarum* spores, PS, SP and LP con-

centrations were significantly elevated ($P < 0.05$) while PC concentration was depressed compared to the untreated control animals, and to the *C. cladosporioides*-treated and saline-treated mice. Exposure to isosatratoxin-F resulted in significantly increased PG and LP concentrations and depressed PI concentration in exposed animals compared to concentrations of these phospholipids in untreated and negative control animals.

FIGURE II:. Phospholipid composition of total lung surfactant from control and treatment mice (* indicates significant difference ($P < 0.05$) from controls (Tukey's Pair-Wise Comparison of Means)).



Figures IIIa-d show the impact of the various treatments on the phospholipid content of 4 surfactant subfractions, P10, P60, P100 and LB from mice. Results show that surfactant subfraction phospholipid concentrations of untreated mice were not significantly different from those saline-instilled mice. Compared to both of the controls, *S. chartarum*-treated mice had significantly elevated SP in the P10 subfraction, phosphatidylserine (PS) in the P60 and P100 subfractions and significantly elevated PC content in the LB fraction. These mice also showed significant depressed PC content in the P10 subfraction. Isosatratoxin-F-treated mice had significantly elevated PC and PE concentrations in the P10 and P60 subfractions and depressed PE concentrations in the P100 subfraction. The *C. cladosporioides*-exposed mice showed significant elevations in PE and PC in the P10 subfraction and LB fraction, respectively, and a significant depression in SP concentration in the LB fraction.

FIGURES IIIA-D: Phospholipid composition of P10 (3a), P60 (3b) and P100 (3c) surfactant subfractions, and Lamellar Body (LB) (3d) fraction in control and treatment mice (*indicates significant difference ($P < 0.05$) from controls (Tukey's Pair-Wise Comparison of Means)).

FIGURE IIIA:

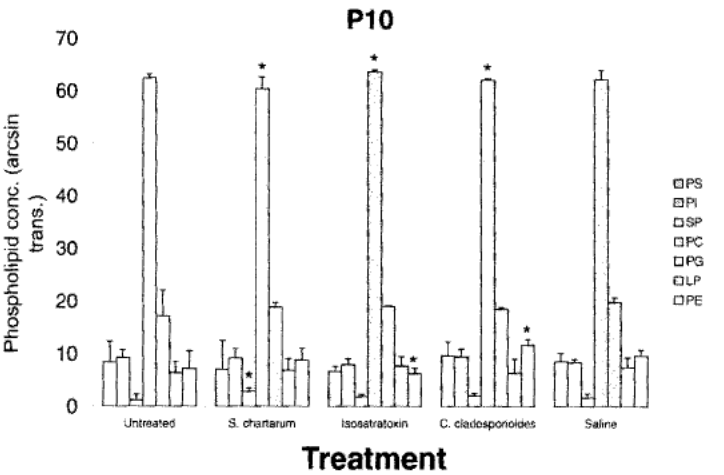


FIGURE IIIB:

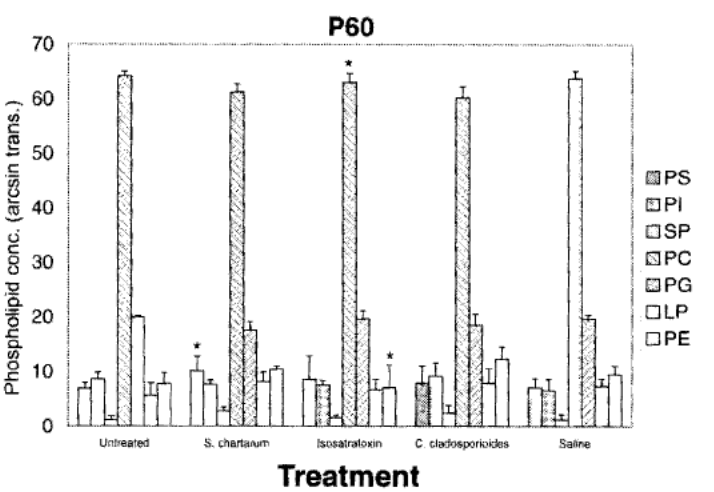


FIGURE IIIC:

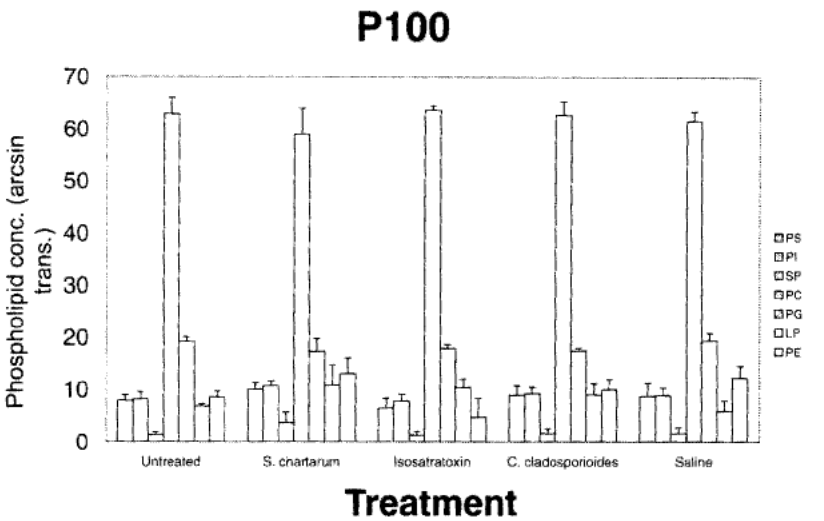
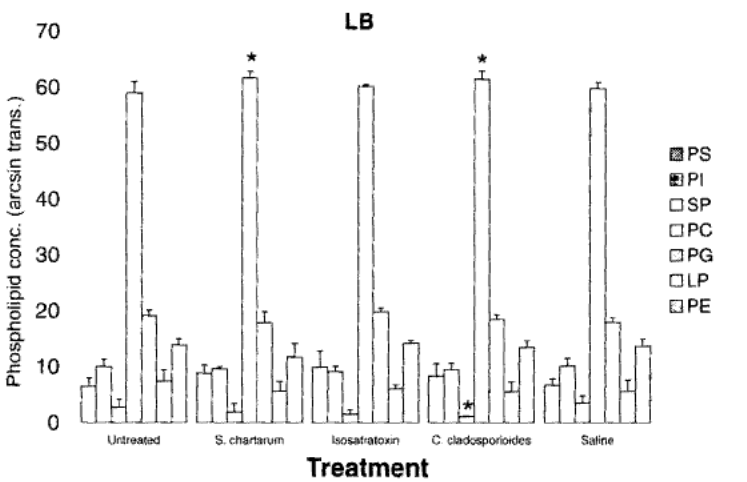


FIGURE IIID:



DISCUSSION

In this study, we have shown that the concentration of the various phospholipids in the different subfractions of untreated and saline-treated mice were not significantly different. This result supports other those of other studies that shows that in healthy animals, the concentrations of phospholipids in surfactant subfractions is similar (Haagsman and Van Golde, 1991). However, in mice exposed either to *S. chartarum* spores, isosatratoxin-F or *C. cladosporioides*, surfactant phospholipid concentrations are altered. Compared to the untreated animals, these changes are manifest both in total surfactant, and in surfactant subfractions.

We are unclear why there are these significant surfactant phospholipid concentration changes. However, there are several possible explanations. Possibly, some of these changes could be due to exposure of alveolar type II to fungal spores and/or toxins, which may damage the cells and result in their reduced ability to synthesize, secrete or catabolize surfactant phospholipids. Increased LP concentrations in the total surfactant pool of *S. chartarum* and isosatratoxin F-treated animals support this notion. Increased levels of this phospholipid are a recognized accompaniment to alveolar type II cell damage due to pollutants impacting on them (Scott et al. 1983, 1986; Scott 1992).

The phospholipid concentration changes observed in the surfactant subfractions of exposed mice may also reflect altered alveolar type II cell membrane dynamics in the exposed animals. Surfactant phospholipids are synthesized in the endoplasmic reticulum of the alveolar type II cell (Jobe and Jacobs, 1984). Biosynthesis of phosphatidylcholine (PC) from choline and diacylglycerol, also involves the transmembranal transportation of choline into the cell via endocytosis and diffusion. As trichothecenes are known to alter cell membrane dynamics and are potent inhibitors of peptidyl-transferase and protein synthesis (Riley and Norred, 1996), exposure of cells to fungal spores and/or toxins, and especially to *S. chartarum* spores and toxin could conceivably interrupt transmembranal transport processes.

Alterations in the activity or concentrations of the enzymes responsible for the biosynthesis of the various phospholipid species from PC, and for the interconversion of the newly secreted surfactant (P10) to metabolically used forms (P60, P100) in the interalveolar space, which is achieved enzymatically, may play important roles. The rate-driving step in PC synthesis, the conversion of cholinephosphate to CDPcholine by CTP-cholinephosphate cytidyl transferase (Post et al. 1984), may be a logical location to examine further the effects of these toxins on phospholipid synthesis (Mason et al. 1998). We recognize that toxic-induced changes in enzyme activity involved in phospholipid synthesis may be easier to interpret if the work is carried out with isolated cells. Therefore, additional work is currently underway to investigate whether the activity of CTP-cholinephosphate cytidyl transferase is modulated due to exposure *S. chartarum* spores, toxin or *C. cladosporioides* that in turn regulates the rate of PC formation and the interconversion of phospholipids in newly-secreted and metabolically used surfactant subfractions using our fetal rabbit model.

It is unclear why phospholipid species concentrations recovered from lavage fluid of animals exposed to *S. chartarum* conidia; isosatratoxin-F and or *C. cladosporioides* were so different. Possibly, these differences observed in our study may be due to the mechanisms of toxicity and the relative toxicity's of the spores of the spores of the two fungal species and toxic substances administered to mice. *Stachybotrys chartarum* produces not only potent trichothecene cytotoxins but also a series of considerably less toxic immunosuppressive substances (Jarvis et al. 1995), some of which can accumulate at high concentrations in *conidia* (Sorenson et al. 1987). It may be that exposure of alveolar type II cells to the combination of trichothecenes in *S. chartarum* spores and the other bio-active metabolites from *C. cladosporioides* is responsible for the observed differences in toxicity seen in the present study.

CONCLUSIONS

This study supports our previous work, which has shown that alveolar type II cells are sensitive to exposure to *S. chartarum* spores and to isosatratoxin F.

Alveolar type II cell sensitivity is manifest by alterations in the composition and concentrations of the phospholipids in alveolar surfactant.

It is clear that additional studies are required to elucidate the mechanisms of the cytotoxicity toward the alveolar type II cells leading to surfactant phospholipid concentration and composition perturbations, in vitro and in vivo.

ACKNOWLEDGEMENTS:

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DIFFERENT METHODS TO CHARACTERIZE MOLDY BUILDINGS

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ABSTRACT

Different methods were evaluated for their potential to show unusual microbial conditions in a building. The buildings studied were schools and offices. The buildings were inspected for visible signs of moisture by a civil engineer. Samples were taken from the air, surfaces and structures. Indoor air concentrations of viable microbes were higher in moldy than reference buildings. This difference could not be seen in the total counts of biological particles. In most cases, the concentrations of microbes on the surfaces were low. In some cases microbial concentrations were high in material samples although no contamination could be seen in surface swab samples from the same damage area. With one sample or one method alone, the conclusion of the mold problem in the building could not necessarily be drawn.

KEYWORDS:

mold, buildings, methods

INTRODUCTION

There are different methods to detect moisture and mold problems in buildings. Microbial concentrations can be characterized in indoor air, on surfaces and in structures. In this study, different methods were validated for their potential to show an unusual microbial situation.

MATERIAL AND METHODS

The buildings studied were two schools and two offices with suspected moisture and mold problems and one reference school and one reference office. The buildings were inspected for visible signs of moisture by a civil engineer. Samples were taken from the air with a six-stage impactor (Andersen), with a single-stage impactor with agar-slide collection system and with

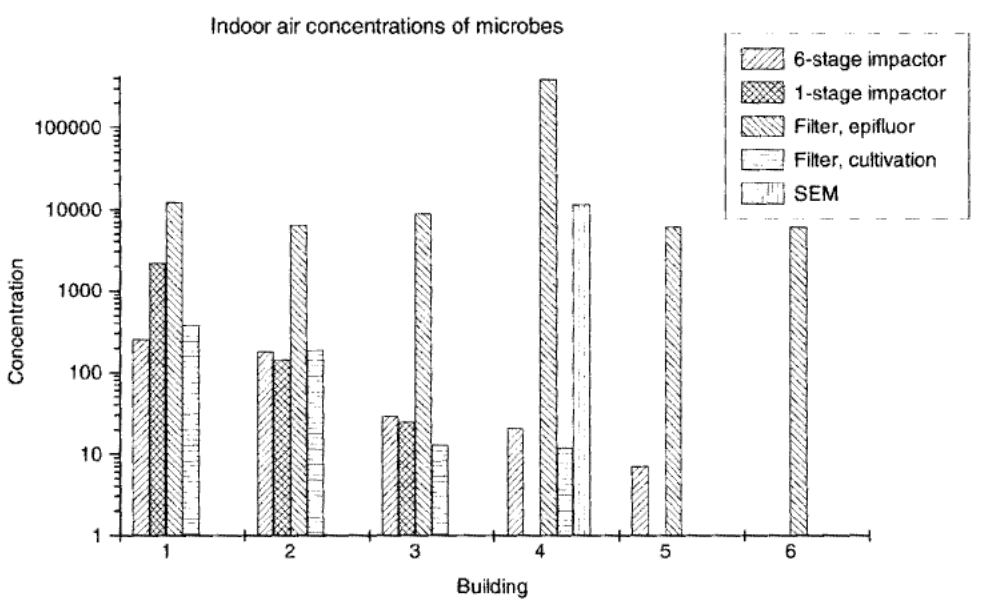
filter collection (analyses by epifluorescense microscope, by Scanning electron microscope (SEM) and by cultivation), from surfaces by swabbing (cultivation and tape samples) and as pieces of materials from structures (cultivation and SEM analyses). The media used were 2% malt extract agar, dichloran 18 % glycerol agar and tryptone-glucose-yeast- agar.

RESULTS

Indoor air concentrations as determined by 6-stage impactor showed increased values compared to the reference (Figure I). This difference could not be seen in the filter samples with direct counting of total biological particles. In one school (#4), an unusual high total count was detected by both epifluorescence and SEM counting but not in the 6-stage impactor samples. Actinomycetes, *Aspergillus fumigatus*, *Eurotium*, *Fusarium*, *Ulocladium* and *Wallemia*, regarded as moisture indicators (Samson et al. 1994), were detected from moisture damaged buildings.

In all but two cases, the concentrations of microbes in the swab samples from the surfaces were low (<100 cfu/cm^2). In one pair of samples, microbes could be seen with direct light microscopy of the tape sample but no microbes could be cultured. This could be an indicator of an old damage, where microbes no longer are culturable. In material samples concentration of viable microbes varied <45 - 5 400 000 cfu/g. In some cases microbial concentrations were high (> 10 000 cfu/g) in material samples although no contamination could be seen in the swab samples from the same damage area. In some materials microbial spores and mycelia were seen by SEM, although nothing could be cultured. This also could indicate an old damage, where microbes no longer are culturable.

FIGURE I: Indoor air concentrations of microbes by different methods. Samples collected in parallel from one room of each building by impactor or filter, (cfu/m^3 by impactor or filter culture methods and spores/m^3 by SEM or epifluorescence microscope). Buildings 1 - 2 index offices, 3 - 4 index schools, 5 - 6 references, respectively.



Conclusions by different methods are presented in Table I.

TABLE I. Conclusions by different methods on whether the result shows damage or not.

Study site	Conclusion of building engineer	Air samples					Surface samples		Material samples	
		6-stage-impactor	1-stage-impactor	Filter			culture	tape	culture	SEM
				epifluor	culture	SEM				
Index sites:										
1 office	+	+	?	-	-	-	-	-	+	+
2 office	+	+	-	-	?	-	?	+	+	-
3 school	+	+	-(?)	-	-	-	-	-	+	+
4 school	+	+ (?)	-	+ (?)	?	+ ?	+	+	+	+
Reference sites:	-	-	-	-	-	-	-	-		
5 office		-								
5 office	-	-	-	-	-	-	-	-		
6 school		+								

Symbols: + =indicative of mold damage, - = no indication of mold damage, ? = can not draw a conclusion. Office 5 and school 6 are the reference buildings.

DISCUSSION

Indoor air concentrations of microbes are not necessarily elevated despite visible microbial damage. Depending on the type of damage, both cultural methods and direct microscopy provided useful information. In order to draw conclusions on the microbial status of the building, characterization of the fungal flora by culture methods is also needed. The detection limit of filter methods were quite high, thus filter methods are not useful in environments with relatively low concentrations of airborne microbes. The results of air samples may indicate a mold problem of a building, but a negative finding does not exclude the possibility of damage.

Surface samples show microbial concentration on the surface. This type of sampling may verify a local contamination, but in some cases, the damage may be behind the surface and thus difficult to detect. In such cases, a sample piece of material may provide more information.

CONCLUSIONS

With one sample or one method alone the conclusion of the mold problem in the building could not necessarily be drawn. Variation of concentrations in the samples was large in both culture and direct microscopy analysis. The methods to define microbial contamination in a building need further validation.

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COMPARATIVE STUDIES OF COLLECTION EFFICIENCY OF AIRBORNE FUNGAL MATTER USING ANDERSEN SINGLE-STAGE SAMPLER AND AIR-O-CELL CASSETTES

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ABSTRACT

The collection efficiency of airborne fungal matter using the Andersen single-stage sampler and Zefon Air-O-Cell cassettes was compared in this study. A total of 814 sets of samples were collected. The correlation coefficient (r) between these two methods was at 0.33 ($p < 0.05$) for total fungal matter and at 0.29 ($p < 0.05$) for *Cladosporium*. The correlation coefficient (r) between the total fungal and *Cladosporium* concentrations collected from Andersen air samples and Air-O-Cell cassettes was at 0.78 ($p < 0.05$) and 0.62 ($p < 0.05$), respectively. *Stachybotrys*-like spores were detected in 74 Air-O-Cell samples. *Stachybotrys chartarum* was detected in 5 Andersen samples. Four sets of samples showed *Stachybotrys chartarum* on both Air-O-Cell and Andersen air samples.

KEYWORDS:

Air-O-Cell cassette, Andersen single-stage sampler (N-6), airborne fungal matter

INTRODUCTION

The Andersen single-stage sampler and Zefon Air-O-Cell cassettes have been widely used for evaluating airborne fungal levels in the indoor environment. The Andersen sampler is used for recovering respirable, culturable airborne fungal spores on suitable fungal media. The Air-O-Cell cassette collects all fungal and other airborne matter. The advantage of using the Andersen sampler is the capability for proper fungal identification. The advantage of using the Air-O-Cell cassettes for total spore counts is the recovery of total airborne fungal matter, including hyphae, conidiophores, and spores, whether viable, dormant, or non-viable.

This study was designed to compare the collection and recovery efficiency between the Andersen single-stage sampler and Air-O-Cell cassettes. *Cladosporium* and *Stachybotrys* are used as indicator organisms for comparisons. *Stachybotrys* spores are atypical in dry, clean buildings, while *Cladosporium* is one of the most common airborne fungi indoors and outdoors (Yang et al. 1993; Lacy, 1981).

MATERIALS AND METHODS

Field samples were collected side-by-side using an Andersen single-stage sampler on 2% malt extract agar (2% MEA) and Air-O-Cell cassettes. The Andersen sampler was calibrated at 28.3 liters per minute (lpm). Sampling time was either for one minute at 28.3 liters (L), two minutes for a total of 56.6 L, or three minutes for a total of 84.9 L. Air-O-Cell cassettes were collected based on the same flow rate as Andersen sampler for 2-4 minutes. A total of 814 sets of samples was collected, from buildings with known fungal contamination and outdoors, between June 1997 and June 1998.

The Andersen single-stage sampler has a 50% cut-off size (the size at which 50% of the particles collected and 50% of the particles penetrate the samples) of 0.65 μm (Jensen et al. 1992). The particle shape and flow rate greatly affected the collection efficiency of the Air-O-Cell cassettes (Willeke, 1998.) At a flow rate of 10 lpm, the cut-size value for oleic acid particles was 3.2 $\pm$ 4% (m; at the flow rate of 30 lpm, the cut-off size value was 1.8 $\pm$ 4% (m. In that study, it showed that aerodynamic properties were important, spiny fungal spores bouncing more than smooth spherical particles.

Andersen samples were incubated at 25°C for seven to ten days. All fungal colonies were enumerated and identified, and total concentrations in colony-forming-units per cubic meter of air (CFU/m^3) were calculated. Air-O-Cell cassettes were mounted with biological dyes (cotton blue or acid fuchsin) and analyzed using the modified NIOSH 7400 fiber counting method. One quarter of the collection band on the slide was analyzed using an Olympus BH-2 compound microscope at 400X magnification. This magnification is capable of detecting the smallest fungal spores (approximately 1.5 - 2 μm). Individual spores, hyphal fragments and conidiophores were counted. Results were calculated and presented as fungal structures per cubic meter of air. Spores were presumptively identified when possible.

RESULTS AND DISCUSSION

The Andersen sampler generally yielded lower fungal concentrations than Air-O-Cell cassettes. The correlation coefficient (r) between these two methods was at 0.33 ($p < 0.05$) for total fungal matter (Figure I) and 0.29 ($p < 0.05$) for *Cladosporium* (Figure II). Due to the low correlation coefficient, it is not practical to use the results from one of these two samplers to estimate the yield of another sampler. The Air-O-Cell samples predictably yielded higher fungal types and concentrations because individual spores, whether viable, culturable or not, and other fungal structures (hyphal fragments and conidiophores) were counted. In addition, spores of some xerophilic fungi were not likely to be detected on the 2% MEA medium. Furthermore, each spore cluster on agar may develop and grow into one single colony.

The correlation coefficient (r) between the total fungal and *Cladosporium* concentrations collected from Andersen air samples and Air-O-Cell cassettes was 0.78 ($p < 0.05$) and 0.62 ($p < 0.05$), respectively (Figure III and IV). *Cladosporium* was not detected in six sets of Andersen and Air-O-Cell cassette samples. 726 of 814 samples (89.2%) of the Air-O-Cell cassettes yielded higher fungal concentrations than Andersen air samples. 737 of 808 samples (91.2%) had a higher *Cladosporium* recovery rate on the Air-O-Cell cassettes than on Andersen samples.

Figure 1: Total fungal structure levels in Air-O-Cell cassettes vs.total fungal conc. in Andersen samples (r= 0.33, n= 792)

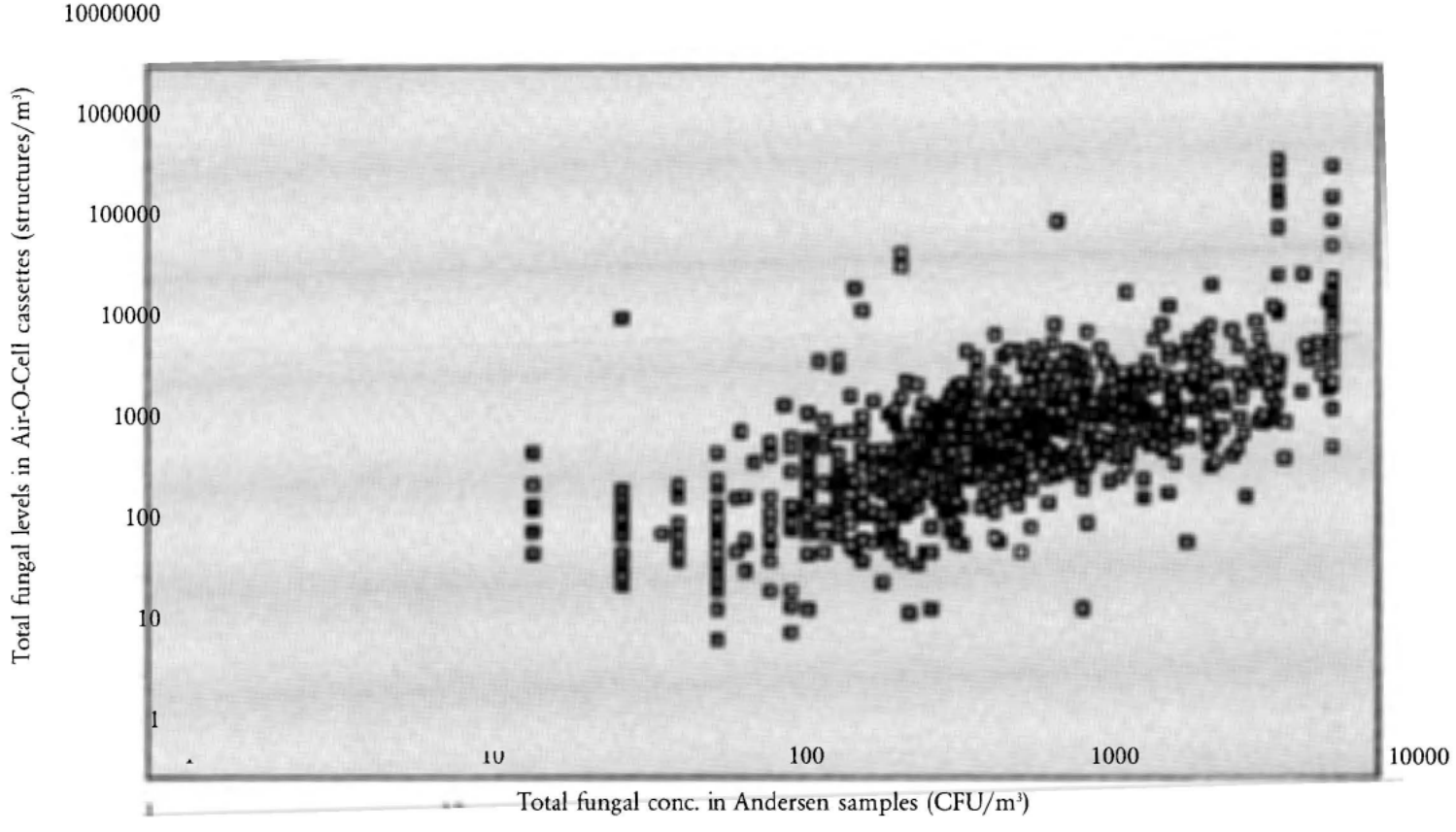


Figure II: *Cladosporium* conc. in Air-O-Cell cassettes vs.*Cladosporium* conc. in Andersen samples (r= 0.29, n= 743)

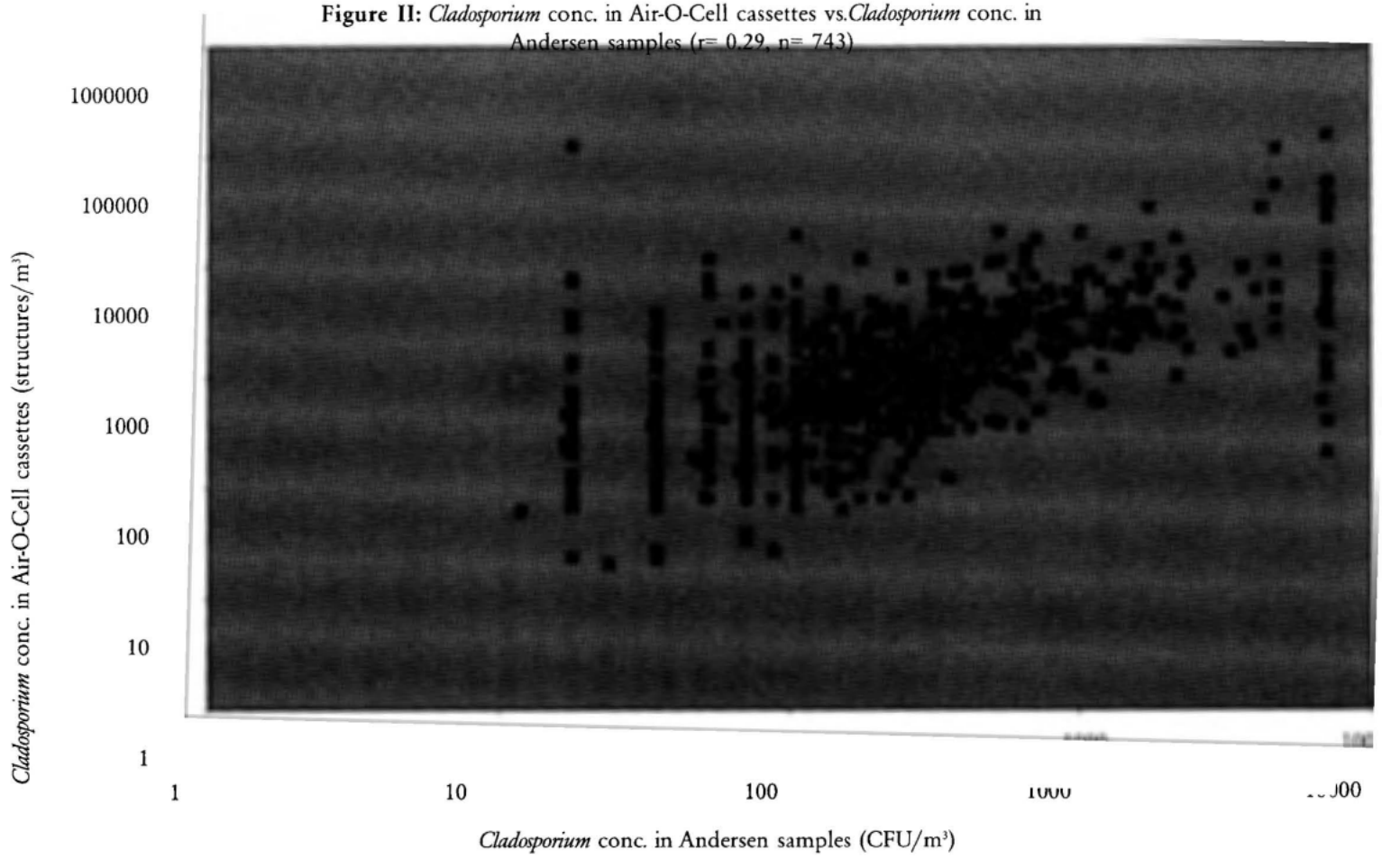


Figure III: Total fungal conc. vs. *Cladosporium* conc. in Andersen samples (r= 0.78, n= 759)

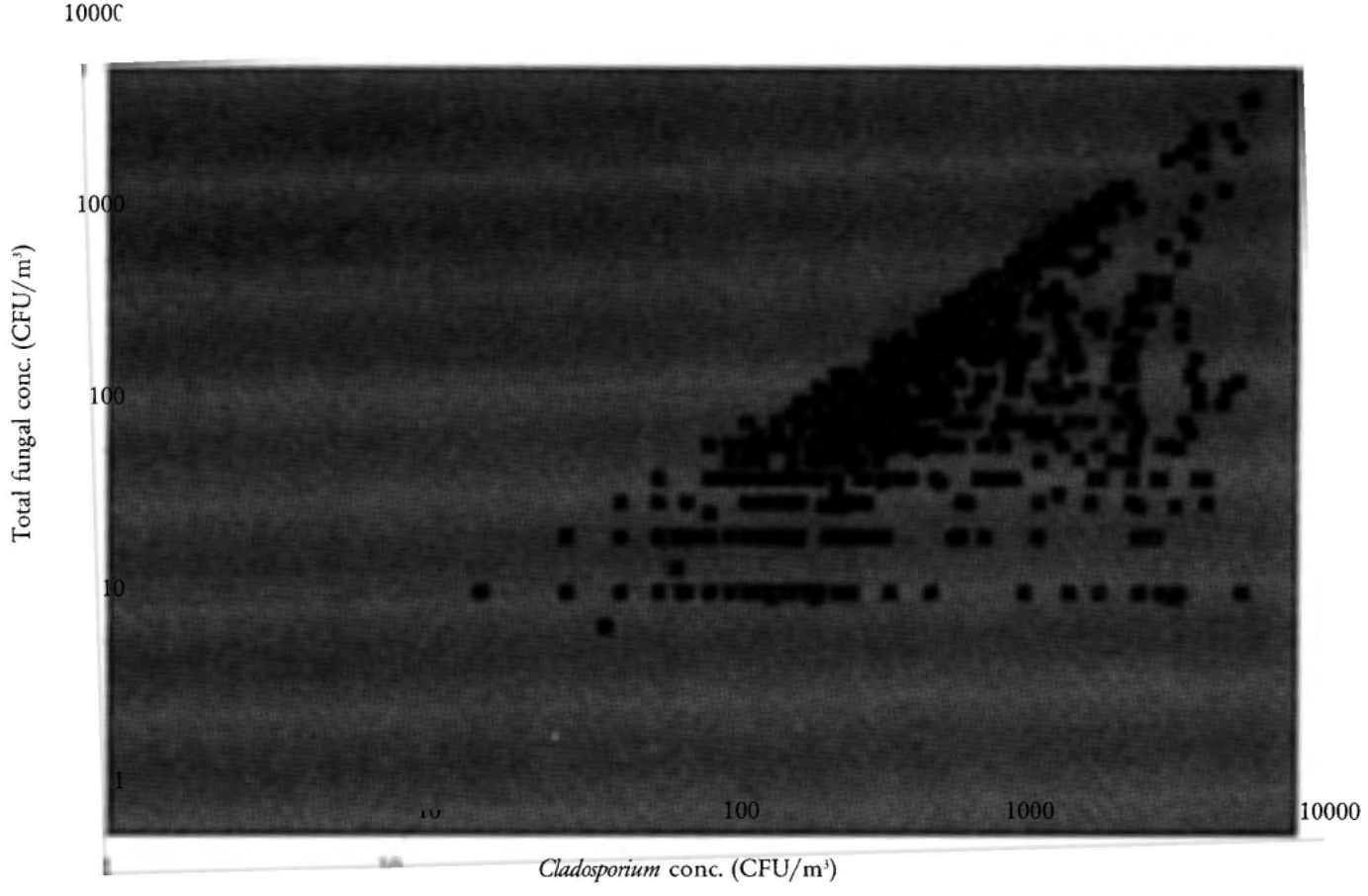
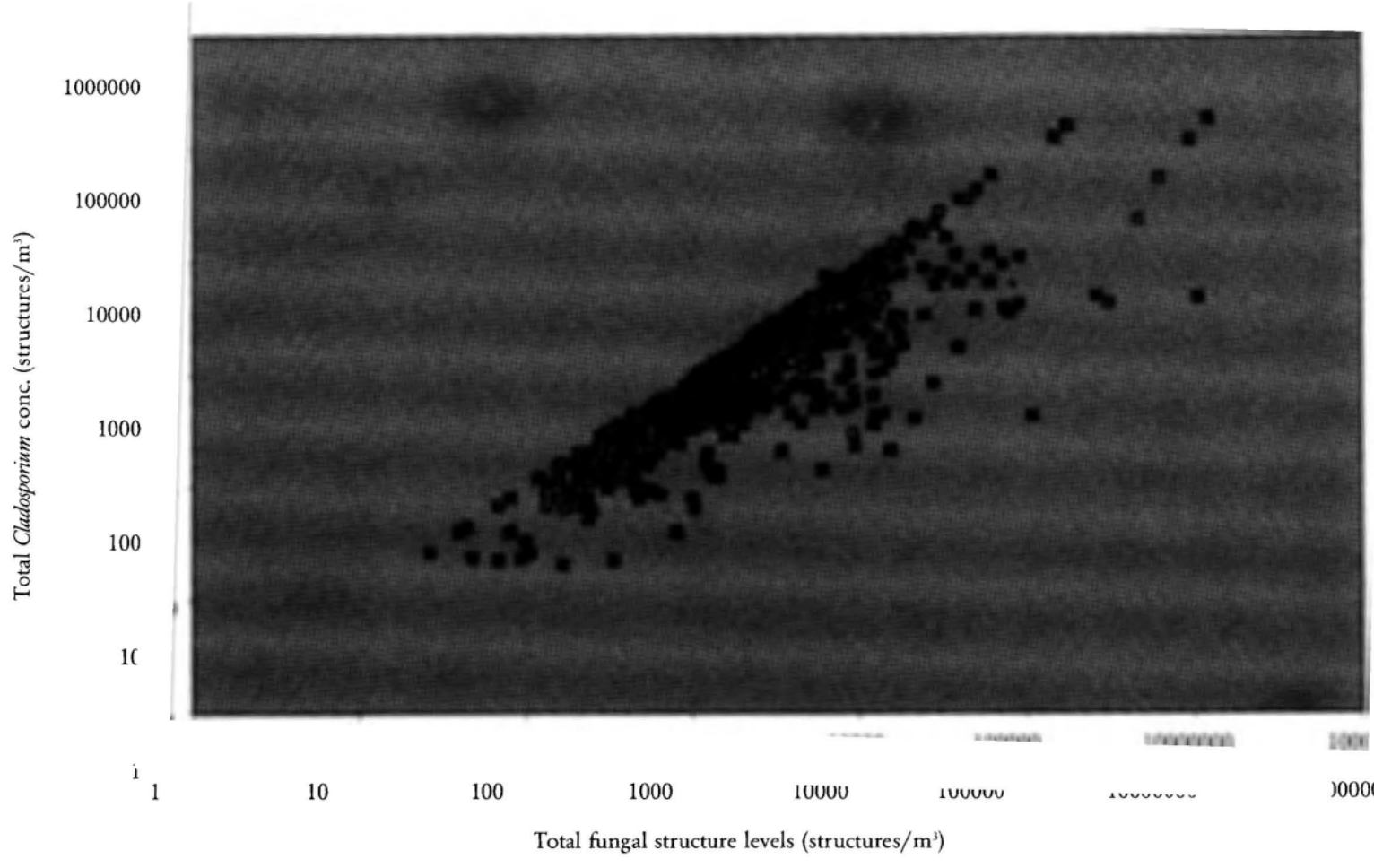


Figure IV: *Cladosporium* conc. vs. total fungal structure levels in Air-O-Cell cassettes (r= 0.62, n= 792)



Stachybotrys-like spores were detected in 74 Air-O-Cell samples. *Stachybotrys chartarum* was detected in 5 Andersen samples. Four sets of samples showed *Stachybotrys chartarum* and *Stachybotrys*-like spores on both Air-O-Cell and Andersen air samples. The higher frequency of detecting *Stachybotrys*-like spores from Air-O-Cell cassettes than from Andersen samplers on 2% MEA was expected because *Stachybotrys* spores may not grow into colonies depending on their viability, the culture media and the presence of other interfering fungi (Tsai et al. 1998). Gravesen et al. (1994) indicated that only a small percentage of the conidia of *Stachybotrys chartarum* are viable in cultures.

Predictably, Air-O-Cell samples yielded higher fungal levels than Andersen samples on 2% MEA. Air-O-Cell samples also had a higher detection rate of *Stachybotrys*-like spores. However, fungal spore identification in Air-O-Cell samples is presumptive at best. Fungal colonies growing on 2% MEA facilitate proper identification of fungi to genus or species levels. Results from total spore counts were a better assessment of airborne fungal spore levels because a significant portion of airborne fungal structures was either non-culturable on the medium used or non-viable. Results in this article suggest that both a culturable method, such as collection with an Andersen sampler, and total spore counts, using the Air-O-Cell cassette or other spore trap samplers, are complementary in the assessment of airborne fungal populations.

CONCLUSION

Results of this study suggest that the correlation coefficient between airborne fungal populations derived from the Andersen impactor and from the Air-O-Cell cassette was significant but not a strong association. Therefore, it is not practical to compare them directly. Andersen culturable sampling provided proper fungal identification while Air-O-Cell cassette samples allowed a better assessment of airborne fungal matter. Fungal concentrations, either total spore or *Cladosporium* alone, derived from Air-O-Cell cassettes were consistently higher than those of Andersen samples. Air-O-Cell samples also had a higher detection rate of *Stachybotrys* than Andersen samples.

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TRICHOTHECENE MYCOTOXINS IN SOME WATER-DAMAGED BUILDINGS

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ABSTRACT

Bulk samples of moldy interior finishes, settled dust, contact inoculated microbiological samples (mixed cultures), as well as pure cultures isolated from indoor environments, were subjected to the qualitative and semi-quantitative simultaneous analysis of 12 trichothecenes. The analysis method was developed as a result of the present study and it includes extraction, sample pre-treatment and reverse-phase HPLC-separation with following tandem mass spectrometric identification and quantitation using electrospray ionization on a quadrupole ion trap mass analyzer.

Similarly to previous studies on fodder or foods, contaminated with trichothecene producing moulds, diacetoxyscirpenol and T-2 toxin were the most prevalent trichothecenes. Apart from these, 3-acetyl-deoxynivalenol, T-2 tetraol, verrucarol and roridin A were occasionally present, particularly in samples contaminated with *Fusaria* or *Stachybotrys spp.* Satratoxins G and H were also found on rare occasions, especially from sites with a severe occurrence of *Stachybotrys spp.* All examined sites were Finnish water-damaged buildings, with confirmed health implications, resulting from fungal propagation.

KEY WORDS:

trichothecene; analysis; indoor air; HPLC/MS

ABBREVIATIONS:

Ace-T-2 (acetyl-T-2 toxin); ESI (electrospray Ionization); CID (collision induced dissociation); DAS (diacetoxyscirpenol); DON (deoxynivalenol); 3-Ace-DON (3-acetyl-deoxy-nivalenol); RDRA (roridin A); SATH (satratoxin H); SATG (satratoxin G); VERA (verrucarin A).

INTRODUCTION

Trichothecenes have been associated with human health hazards for decades (Kilbourne-Matossian, 1989). These are typically multi-toxins, affecting many systems (Hall and Rumack, 1998). To date, well over a hundred trichothecenes have been identified, almost exclusively

from fungal cultivations, including genera belonging to *Fusarium, Trichothecium, Cylindrocarpon, Myrothecium, Trichoderma, Verticinosporum, Cephalosporium and Stachybotrys* (Ueno,1983; WHO, 1990). The majority of the reported producers belong to the *Fusarium* (anamorph *Gibberella*) genus (WHO, 1990). *Fusarium, Stachybotrys* and other trichothecene-producing moulds have most frequently been found to infect grains (Ueno, 1983; WHO, 1990), but with the establishment of new laboratory practices in environmental laboratories, they have been isolated from indoor environments as well (Croft et al. 1986; Andersson et al. 1997; Flannigan et al. 1991). When potential trichothecene-producers are present in indoor environments, one would expect to encounter health problems similar to those previously encountered with people handling trichothecene-contaminated seed. Indeed, in recent years, *S. atra*, in particular, has been isolated from building materials and air samples in buildings with moisture problems, the residents of which suffer from very similar symptoms, such as cough, irritation of eyes, skin and respiratory tract, headache and fatigue (Croft et al. 1986; Johanning et al. 1993; 1996; Hodgson et al. 1998).

However, the most important genus with respect to trichothecene production is *Fusarium*. This particular genus has not frequently been encountered in indoor climates. Certainly, part of this could be due to isolation and cultivation techniques, rather than to the true prevalence of these species. Research on the microbial flora of plants has shown that Fusaria present in native samples require special measures in order to be detected. Traditionally, species of *Fusarium* have been isolated on starch-based media, containing particular fungicides to inhibit the growth of more rapidly sporulating mucoraceous fungi, such as *Aspergillus, Penicillium* and *Cladosporium* (Andrews and Pitt, 1986; Abildgren et al. 1987). With seeds, it appears that *Fusaria* grow well when applying intact seeds on modified Czapek-Dox agar plates, containing iprodione and chloramphenicol and subjecting the plates to light with spectra resembling day-light (Abildgren et al. 1987; Tuomi et al. 1995). It seems therefore unlikely, that all *Fusaria* present in indoor environments will become detected on spread plates with standard techniques. Therefore, even when no *Fusaria* are found upon isolation, there is a possibility that trichothecenes and other metabolites of *Fusaria* are present in buildings suffering from severe moisture problems.

The present study was undertaken with the intention of gathering relevant data about the occurrence and concentration of common fungal trichothecenes in Finnish indoor environments and with the specific aim to develop methodology that can be used to extract and quantitate a wide spectrum of trichothecenes from samples derived from both building materials and microbial growth media. This involved large numbers of samples with small amounts of extractable material, containing minute amounts of toxin. With the above mentioned microbiological difficulties in mind, whenever possible, we tried to obtain either bulk samples of moldy surfaces, or settled dust. This was not, however, possible in many of the examined sites, since surfaces carrying visible growth are usually well hidden and sufficient amounts of dust can be hard to obtain. In such cases, we used contact inoculation of agar slides on particularly dusty surfaces, i.e. surfaces where dust had been depositing for several weeks. Air sampling was not considered in this study.

METHODS

Sample composition

The method has been applied to bulk samples of moldy interior finishes, ranging from paper-covered gypsum board to carpeting and wooden material. Further, contact inoculated Hygicult® agar slides (Xygen diagnostics, Inc., Ontario, Canada) and fungal isolates grown on solid malt-extract agar (Hispanlab, Madrid, Spain) plates were analyzed, as were samples of settled dust. Dust samples were collected by either suction to air filters (Millipore AAWP 0037, Millipore, Inc., Bedford, Massachusetts) or by dusting of surfaces.

The Hygicult® agar slides contained mixed cultures, which were allowed to grow until exhaustion, in the dark, at room temperature (25°C). Slides were stored at room temperature in closed containers for up to 3 years prior to analysis. Slides were inoculated by pressing the agar surfaces against moldy or dusty interior finishes.

Spread plates with pure cultures were incubated, in the dark, at 25°C for 1-2 weeks. Plates were stored at 5°C for up to two weeks prior to analysis. Pure cultures had been isolated from mixed cultures obtained from indoor environments by swabbing.

Identification of fungal species

The fungi were identified, based on their morphological characteristics at the City of Helsinki Environment Center, Helsinki, Finland; the Finnish Institute of Occupational Health, Uusimaa Regional Institute, Helsinki, Finland; and the National Veterinary and Food Research Institute, Helsinki, Finland.

Preparation and analysis of samples

The method of analysis has been outlined by the authors in an upcoming article (Tuomi et al. 1998).

RESULTS AND DISCUSSION

Prevalence of toxins

Altogether 189 samples were analyzed between March and July 1998. Of the 48 trichothecene positive samples encountered, 34 contained either or both of DAS and/or T-2 toxin. T-2 toxin was more common than DAS, but this might be the result of a lower detection limit (Tuomi et al. 1998) rather than a true reflection of prevalences. Apart from these, 3-acetyl-deoxynivalenol, verrucarol, T-2 tetraol and roridin A were found in some of the sites (Table I). Satratoxins G or H were found in three sites, in two of which, a severe occurrence of *Stachybotrys spp.* had been established (Table I). No findings of T-2 triol, HT-2 or verrucarin A were made. In addition, 141 samples with medical symptoms suggesting towards trichothecenes but with negative results upon chemical analysis were encountered. Unfortunately, we were not able to include DON and 3-Ace-DON in the analysis scheme until late on in the study. The data in Table I are therefore not representative of the prevalence of these compounds. Subsequently to the presented study, we have included Acetyl-T-2 toxin (Ace-T-2), cit-

rin, Sterigmatocystin, ochratoxin A and Nivalenol in the present analysis scheme. Of these, we have so far detected Ace-T-2, Sterigmatocystin and citrin from native samples, derived from indoor environments (results not shown).

TABLE I: Sources of trichothecenes. Sites are indicated by asterixes[1-11]. Dates of sampling are indicated.

Sample type	Source	Concentrations of detected mycotoxins	Identified species /Cytotoxicity on rabbit kidney cells
Mineral wool[1, 1997]	Wall isolation	T-2 toxin: 0.33 ng/cm^2	na/++
Mineral wool[1, 1997]	Wall isolation	T-2 toxin: 0.014 ng/cm^2	na/++
Plywood[1, 1997]		T-2 toxin: 9 pg/cm^2	S. spp./+
Plasterboard[1, 1997]	Inside roof covering	Verrucarol: 8.8 ng/cm^2 T-2 toxin: 0.11 ng/cm^2	S. spp./-
Mineral wool[1, 1997]	Wall isolation	T-2 toxin: 0.069 ng/cm^2	na/+
Mineral wool[1, 1997]	Wall isolation	T-2 toxin: 0.069 ng/cm^2	na/+
Plywood[1, 1997]		Verrucarol: 23 ng/cm^2	na/-
Cardboard[2, Nov. 1995]	Wall isolation, child rens water-play room in day-care center	DAS: 6.4 µg/g T-2 toxin: 1.6 µg/g	*S. atra*
Framework stock,[2,Nov 1995]	Day-care center, wall covering in child ren's water-play room	DAS: 2.0 µg/g T-2 toxin: 0.84 µg/g	*S. atra*
MEA-agar plate[2, Nov 1995]	Day-care center, wall covering	DAS: 16 ng/ml SATG: 400 ng/ml SATH: 29 ng/ml RDRA: 1.9 ng/ml	*S. atra*
Settled dust particles collected by suction to fiberglass filter[2, Nov 1995]	Day-care center, baseboard, inside wall towards west	DAS: 5.9 µg/g T-2 toxin: 0.30 µg/g	na
Settled dust particles collected by suction to fiberglass filter[2, Nov 1995]	Day-care center, baseboard, inside wall towards east	DAS: 0.11 µg/g T-2 toxin: 0.015 µg/g	na
Hygicult agar[2, Nov 1995]	Day-care center, next to baseboard	T-2 toxin: 28 ng/ml	na, mc
Hygicult agar[2, Nov. 1995]	Day-care center, baseboard	T-2 tetraol: 63 ng/ml T-2 toxin: 1.2 ng/ml	na, mc
Hygicult agar[2, Nov, 1995]	Day-care center, baseboard	T-2 toxin: 29 ng/ml	na, mc
Paper-covered gypsum board,[3 May 1998]	Wall-covering	DAS: 0.29 µg/g T-2 toxin: 0.038 µg/g	na
Gypsum board with remains of tile[4, May 1998]	Bathroom wall-covering	DAS: 11 ng/g SATG: 1.4 ng/g T-2 toxin: 2.2 ng/g	*S. atra* 4.1x10^6 cfu/g *Acr. spp.*3.5x10^5 cfu/g *P. spp.* 4.5x10^3 cfu/g *A. sydowii* 9.0x10^3 cfu/g
Gypsum board[5, May 1998]	Bathroom wall-covering	DAS:0.083 µg/g	na

TABLE I cont.

Sample type	Source	Concentrations of detected mycotoxins	Identified species /Cytotoxicity on rabbit kidney cells
Hygicult agar[6, Dec. 1995]	Deposited dust on laminated cupboard in office	DAS: 2.1 ng/ml T-2 toxin: 0.26 ng/ml	na, mc
Hygicult agar[6, Dec. 1995]	Deposited dust on laminated cupboard in office	DAS: 1.7 ng/ml T-2 toxin: 0.8 ng/ml	na, mc
Hygicult agar[6, Dec. 1995]	Deposited dust on laminated cupboard in office	DAS: 13 ng/ml T-2 toxin: 5.8 ng/ml	na, mc
Hygicult agar[6, Dec. 1995]	Deposited dust on plastic pipelines in office	DAS: 8.4 ng/ml SATG 7.1 ng/ml	na, mc
Hygicult agar[6, Jan 1996]	Deposited dust on PVC-floor in office	T-2 toxin: 3.5 ng/ml	na, mc
Hygicult agar[6, Febr. 1996]	Deposited dust on painted metal cupboard in office	DAS: 18 pg/ml T-2 toxin: 0.4 pg/ml	na, mc
Hygicult agar[6, Febr. 1996]	Deposited dust on painted wooden shelf in office	DAS: 43 ng/ml T-2 toxin: 6.8 ng/ml	na, mc
Hygicult agar[6, Febr. 1996]	Deposited dust on painted wooden shelf in office	DAS: 20 ng/ml T-2 toxin: 3.8 ng/ml	na, mc
Hygicult agar[6,Febr. 1996]	Deposited dust on painted wooden shelf in office	DAS: 20 ng/ml T-2 toxin: 2.0 ng/ml	na, mc
Indoor dust from office[6, June 1998]	Dust collected in air cleaner filter during a 3 month period + dust collected on water-heated air thermostat	3-Ace-DON > 0.1 pg/g (quantitative result na)	Fu. spp.
Hygicult agar[7, March 1996]	Water-damaged painted gypsum board in office	T-2 tetraol: 0.1 µg/ml	na, mc
Hygicult agar[8, March 1996]	Deposited dust on computer-cover in office	Verrucarol: 9.5 µg/ml	na, mc
Hygicult agar[8, March 1996]	Water-damaged painted gypsum board in office	Verrucarol: 0.20 µg/ml	na, mc
Hygicult agar[9, May 1998]	Soil surface of ornamental flower in office	Verrucarol: 230 µg/ml	na, mc
Soil from ornamental flower[9, May 1998]	Office-building	T-2 toxin:54 ng/ml RDRA: 0.14 µg/ml	Fu. spp
Soil from ornamental flower[9, May 1998]	Office-building	RDRA: 48 ng/ml	Fu. spp.

TABLE I cont.

Sample type	Source	Concentrations of detected mycotoxins	Identified species /Cytotoxicity on rabbit kidney cells
Soil from ornamental flower[9], May 1998	Office-building	T-2 toxin: 95 ng/ml RDRA: 180 ng/ml	Fu. spp
Hygicult agar[10], Febr. 1997	Interior surface in day-care center	Verrucarol:18 µg/ml	S. spp
Hygicult agar[10], Febr. 1997	Interior surface in day-care center	Verrucarol: 65 µg/ml	S. spp
Hygicult agar[11], Oct. 1995	Water-damaged wall covering in day-care center	RDRA: 16 ng/ml	na, mc
Hygicult agar[11], Oct. 1995	Deposited dust on cup-board in day-care center	T-2 toxin: 4.2 ng/ml RDRA: 3.0 ng/ml	mc, S. spp
Hygicult agar[11], Oct. 1995	Painted wooden wall in day-care center	RDRA: 4.2 ng/ml	na, mc
Dust-sample[11], Oct. 1995	Deposited dust on floor in day-care center	T-2 toxin: 1.5 µg/g RDRA: 1.2 µg/g	na

na= not available; mc= mixed culture; Acr. spp = Acremonium spp.; A. syowii = *Aspergillus* sydowii; Fu. spp. = *Fusarium* spp.; P. spp. = *Penicillium* spp.; S.spp.= *Stachybotrys* spp.

Toxins and symptoms

All samples were derived from workplaces, i.e. factories, schools, day-care centers, offices and the like, where the company doctor or health care unit had encountered symptoms suggesting pronounced fungal propagation due to water damage or construction deficiencies. Typical irritatory symptoms, which we were looking for when selecting the target sites included: irritation of throat and eyes, dryness of mucous membranes and skin, spotted skin, headache and fatigue, inflammation of trachea and cough, hoarse throat and fading voice, and nausea and dizziness. These are all signs of a different symptom path than the allergic route commonly associated with indoor fungi. In spite of this, the trichothecene containing samples could not as a rule be linked to a medically diagnosed work-related illness (results will be presented elsewhere). This might not be all that surprising, however, considering that upon short term exposure trichothecenes exert ambiguous effects common to many other irritatory states and, further, that reports on the long term effects of trichothecene exposure are rare (Hall and Rumack, 1998; WHO 1990). Also, most of the sites were in such condition, that they have since been renovated and, hence, the symptoms have disappeared. In some cases, however, following renovation, irritatory symptoms have reappeared after some time, suggesting that renovation succeeded in removing the consequence, rather than the original source, of the contaminating factor.

Critical for the exertion of symptoms is the actual concentrations of toxins in indoor air. Our samples were derived with varying techniques, the lot of which did not include air sampling on filters, in a setup that would enable estimates of concentrations in air. In dust, we encountered concentrations between 15 ng/g to 6 µg/g (Table I). Previously, in agricultural surroundings,

trichothecene concentrations of 0.1-1 μg/g dust (Lappalainen et al. 1996) have been reported. In laboratory settings, Sorensen and co-workers (1987) found concentrations in the 10 μg/g dust-range. However, these findings, while indicative of the concentration ranges in dust, do not facilitate any comparisons of airborne toxin concentrations.

CONCLUSION

All compounds could be purified and quantitated simultaneously, with only a few, not very labor and time consuming treatment steps. It clearly seems therefore that quadrupole ion trap tandem mass-spectroscopy with positive electrospray ionization is well suited for the detection and quantitation of trichothecene mycotoxins, separated by reverse-phase HPLC.

According to present day literature, the most frequent natural contaminants are nivalenol, deoxynivalenol, diacetoxyscirpenol, HT-toxin, nivalenol and T-2 toxin (WHO, 1990). The present study included too few sites - sampled with differing techniques - to say anything conclusive about the prevalence of the measured compounds. More importantly, however, the study establishes that trichothecenes familiar from agricultural surroundings do frequently occur in the indoor environment of severely water-damaged building sites.

From the concentrations encountered with different measuring techniques, it would seem that when screening for mycotoxins, bulk samples of interior finishes or dust samples are preferable to growth media of inoculated fungi - whether agar plates or slides. As pointed out by Pasanen et al. (1993), among others, sampling of mycotoxins is preferably done with analytical methods that are independent of the ability of mycotoxin-carrying spores to germinate. Undoubtedly, such an approach will uncover what is really present on materials and in deposited dust. The drawback with using construction materials is that in reality, viable *mycelia* can be hard to find, as visible growth of fungi occur mostly behind walls and isolation plates. This, certainly, will lead to many false negative results, but this is just as true with any other measuring technique. In cases where no visible growth can be located, dust sampling is one possibility. Another possibility is the approach taken in the present study, i.e. contact inoculation of agar slides on dusty surfaces. This is, however, associated with apparent limitations. One is the possibility that mycotoxin-carrying spores do not germinate. Another is that it is difficult to obtain pure cultures and, hence, to identify individual species from such slides. Also, agar slides support less biomass than the agar plates more commonly used in indoor investigations and hence, potentially less mycotoxins. In any case, evaluations of mycotoxin concentrations in air can only be done through air sampling on filters, for prolonged periods of time.

In conclusion, before predicting the health implications of trichothecenes present in indoor samples, it has to be found out whether the compounds transmit to air and whether they are, as a result of such transmission, inhaled and absorbed by persons breathing the air. Also, presently, it is hard to predict what levels of trichothecenes or other mycotoxins in indoor air can be deemed as unhealthy. Still, even though finding trichothecenes in a building does not in itself prove that the building is unhealthy to be and work in, we feel that it is advisable to try and locate and remove the source of contamination and consider whether or not the site should be renovated.

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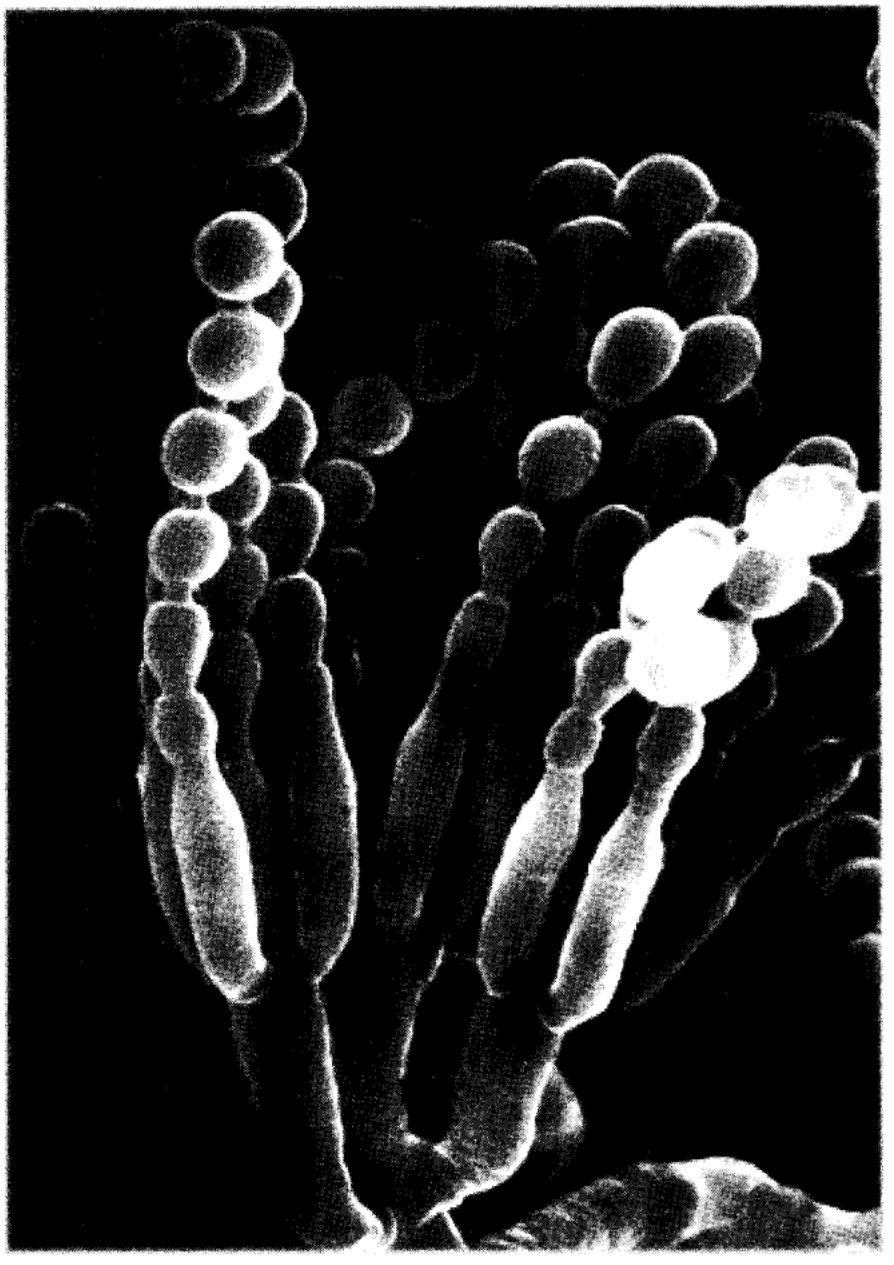

Penicillium chrysogenum, EM 5,800 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands

Chapter 4

Applied Research

SARCOIDOSIS AND EXPOSURE TO OCCUPATIONAL AND ENVIRONMENTAL AGENTS

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ABSTRACT

An index patient with sarcoidosis that appeared reversibly related to a school generated a case-control study. All 59 patients seen between 1992 and 1997 in a pulmonary division, and unmatched controls from orthopedic surgeons, received two copies of a self-administered questionnaire inquiring about occupational and environmental histories. Thirty-one (52.2%) of the patients and ninety-four (32.4%) of the controls responded. Patients were significantly more likely to have been exposed to inorganic dusts (odds ratio [OR] 3.48; 95% confidence interval [95%CI]: 1.02 - 11.80), molds (OR 9.8; 95%CI: (0.98 - 98.40) and solvents or oils in the work place (OR: 9.8; 95%CI: (1.60 - 48.0) than controls. They described exposure at home through moldy basements (OR: 2.37; 95% CI: 1.0, 5.8) or mold on bathroom walls (OR: 5.69; 95% CI 1.3-25.6) more frequently than controls. Sarcoidosis may represent a disease at least in part attributable to occupational and environmental exposures.

KEY WORDS:

sarcoidosis, bioaerosols, indoor air, environmental disease, occupational disease, moisture

INTRODUCTION:

Newman and Rose (Newman et al. 1997) reviewed prior attempts at defining the etiology of sarcoidosis and suggested that either shared exposures or person-to-person transmission are likely factors contributing to the development of sarcoidosis. Subsequently, an outbreak on an aircraft carrier (Jajosky, 1998) provided further support to these hypotheses but could not distinguish between them. Because of a teacher with sarcoidosis that appeared reversibly related to work in a wet school, the authors undertook a case-control study to test the hypothesis that patients with sarcoidosis were more likely to be exposed to molds, moisture, and vegetable dusts in the work place and at home than controls.

METHODS:

POPULATION

Cases: All patients seen at the University of Connecticut Division of Pulmonary Medicine between 1992 and 1997 with a primary diagnosis of sarcoidosis were eligible and received two mailings of a questionnaire. Patient files were reviewed to ensure they met objective criteria for sarcoidosis (Winterbauer et al. 1973; Sulavik et al. 1993):

- symptomatic bilateral symmetric hilar lymphadenopathy with or without mediastinal lymphadenopathy in the right clinical context (age, non-HIV etc.).
- Non-caseating granuloma on biopsy with evidence of clinical disease in one organ.
- "Lambda/panda sign" with a compatible chest x-ray and suggestive clinical findings.

CONTROLS:

Controls consisted of patients seen in the offices of three orthopedic surgeons at three hospitals who had previously been selected as controls in a 1994 (Mullen et al.1998).

QUESTIONNAIRE

The questionnaire, developed by Scott (Scott.et al. 1990) and modified by Mullen (Mullen et al. 1998) inquired about a work history (employer, industry, job title, job description), an exposure inventory (lists of exposures and their associated duration), and questions about residential exposures (evidence of moisture and water damage, kitchen and bathroom exhaust, and basement descriptions).

DATA CODING AND ANALYSIS

A work history was developed for each respondent from the questionnaire section inquiring about work history. Exposure estimates were constructed by an occupational physician (MJH) and an occupational safety and health engineer (CO), with the former was blinded to case-control status. Where these two disagreed, a certified industrial hygienist was consulted. In this study only "any exposure" was analyze because of the small sample size. This approach has been used in similar studies (Hodgson et al. 1991; Brancati et al. 1993; Mullen et al. 1998) by one of the authors.

Data were analyzed using SPSS Release 5.0.2 in a Windows environment (Norusis, 1992). Where data did not appear normally distributed, non-parametric comparison techniques were used. An alpha level of 0.05 was defined as significantly different from chance.

Exposure data concordance was evaluated using kappa statistics. Where differences were seen for the whole population, data were scrutinized again after stratification on that pertinent variable and by logistic regression analysis controlling for that variable.

RESULTS:

Data on ethnicity and race for the case- and control populations are not available. Of the 59 cases eligible to participate, 31 (52.5%) completed and returned the questionnaire; twenty-four

(24) did not respond after the second mailing; and four (4) mailings were returned by the postal service as undeliverable. Of the 31 respondents one was disqualified because insufficient data were provided to construct an occupational history. Another respondent was identified as the index case after the fact and was also disqualified. The remaining 29 cases were included in the study. In the earlier study from which the controls were drawn 94 (32.4%) of the 290 eligible controls responded. Of the 94 respondents, eight (8) were disqualified because of insufficient data, leaving 86 controls for this study.

Significant differences were observed between the case and control populations. Cases were younger (mean age cases: 52.7 controls: 67.1; $p<0.001$), more likely to be male (51.7% as opposed to 31.45; $p<0.05$), and African Americans (28.6% of cases; no controls: $p<0.0001$). Among only Caucasian respondents, cases were still significantly younger (58.3 vs. 67.3; $p=.022$), but gender was no longer significantly different. In both sets of analyses, there were no differences in ever employment status or employment duration.

Cases and controls did not differ in environmental exposures to gardening, pesticides, pets in the home, carpeting, heating source or distribution, or age of residence (Table I). Cases were

TABLE I: Self-reported Environmental Exposures, all participants.

	Cases (n=29)	**Controls (n=86)**	**OR (CI)**	**P***
Regular smokers	15 (51.7%)	45 (52.3%)	0.97 (.42-2.3)	Ns
Alternative heat	12 (41.4%)	39 (45.3%)	0.85 (.36-2.0)	Ns
Forced air heat	8 (27.6%)	24 (28.6%)	0.95 (.37-2.4)	Ns
Pet animals	22 (75.9%)	68 (79.1%)	0.83 (.31-2.3)	Ns
Gardener	21 (72.4%)	56 (65.1%)	1.41 (.56-3.6)	Ns
Pesticide use	10 (34.5%)	26 (30.6%)	1.19 (.49-2.9)	Ns
Age of home#	40.4 (24.0 yrs	43.1 (36.0 yrs	N/A	Ns
Carpeting	24 (82.8%)	69 (80.2%)	1.18 (.39-3.6)	Ns
Kitchen exhaust	14 (50.0%)	44 (51.8%)	0.93 (.40-2.2)	Ns
Moisture problems	16 (55.2%)	30 (34.9%)	2.30 (.98-5.4)	Ns
Bath walls	5 (7.2%)	3 (3.5%)	5.69 (1.3-25.6)	.025
Shower curtain	5 (17.2%)	13 (15.3%)	1.15 (.37-3.6)	Ns
Basement	14 (51.9%)	25 (31.3%)	2.37 (.97-5.8)	Ns

*: p-value associated with chi-square test
#: t-test

significantly more likely to describe any moisture problem in the home, visible mold growing on bathroom walls, and moisture problems in the basement. When African Americans were excluded from the analyses, the associations were numerically stronger, i.e., odds ratios higher, two thirds of cases and one third of controls reporting moisture problems in the home ($p=.034$).

The relationship between self-assessments of exposure by the subjects and by the investigators ratings was scrutinized. Kappa statistics comparing exposure to molds, solvents, and any inorganic dusts were above 0.45, suggesting good agreement. Table II presents the association of various occupational exposures with sarcoidosis for all subjects. Exposure to molds and organ-

ic solvents appeared significantly associated with disease. The associations with exposure to mold was weaker and the association with solvent exposure still elevated but no longer statistically significant when the analysis was repeated using data from Caucasian respondents only. Similarly, age stratification (above and below the median age) led to disappearance of the statistical significance although the odds ratio remained elevated.

TABLE II: Estimated Occupational Exposures, all participants.

	Cases Exposed N	%	Odds ratio	95% confidence interval
SiO2	2	6.9	0.93	(0.84 - 1.03)
Ni/Cr/Steel	1	3.4	0.48	(0.05 - 4.13)
Be	1	3.4	0.97	(0.90 - 1.03)
Other inorg dusts	3	10.3	1.30	(0.31 - 5.40)
Any inorg dusts	6	20.7	3.48	(1.02 - 11.8)
Birds	1	3.4	0.97	(0.90 - 1.03)
Molds	3	10.3	8.8	(1.83 - 47.48)
Wood dust	1	3.4	3.03	(0.18 - 50.10)
Other org dusts	1	3.4	0.99	(0.10 - 9.89)
Any org dusts	3	10.3	1.87	(0.42 - 8.36)
Org solvents	5	17.2	8.75	(1.60 - 48.0)
Chlorinated solvent	0			
Nox	0			
Plastics	1	3.4	0.99	(0.10 - 9.89)
Oils	2	6.9	1.20	(0.22 - 0.50)
Welding fume	0			
Pesticides	1	3.4	1.01	(0.99 - 1.04)
Animals	2	6.9	1.52	(0.26 - 8.76

A single patient (older Caucasian male), and no controls, described exposure to beryllium. Twenty (69%) of the sarcoidosis patients had at least one pertinent exposure at home or at work as compared with 37% of the controls (p=0.029). Three of the six African-Americans cases had exposure to at least one exposure of concern: one was exposed to molds in his work place, one had a moldy basement and mold on bathroom walls together with solvent exposures at work; and one had mold on bathroom walls alone. One of the Caucasian patients described mold exposure in the work place and ten at home (two moldy bathroom walls and eight moldy basements)

DISCUSSION:

This study suggests that sarcoidosis is associated, among others, with bioaerosol exposure in the home and the workplace. Misclassification from hypersensitivity pneumonitis (HP) is unlikely because the criteria used to classify disease are standard. Bioaerosols are clearly associated with HP. Associations with organic solvents and inorganic dusts are equally interesting, though less widely known, but plausible (Newman et al. 1997). These are consistent with risk

factors identified for other forms of interstitial lung disease (Iwai et al. 1994; Scott et al. 1990; Hubbard et al. 1996). The results provide support to the hypothesis that the spectrum of clinical forms of lung disease may result from a genetically-based differential response to a common set of antigens (Lympany et al. 1997).

Several methodological weaknesses must be considered. Only 56.4% of the patients responded. The non-respondents may have had higher rates of exposures, a differential response bias, but this would have lead to an deflation of the measures of association, as African-Americans and less affluent individuals, who are more likely to be exposed to hazardous exposure, are generally less likely to respond to such surveys leaving less highly exposed subjects as cases. Second, self-administered questionnaires are always deemed slightly suspect. Nevertheless, there was good agreement between the exposures identified by subjects themselves and those coded by the investigators on the basis of general knowledge of trades and occupational categories. This too, therefore, is not of great concern. The relatively small sample size does bias against finding anything but very strong associations and limits the generalizability of this study. Most importantly, no African-Americans existed among our control population. Nevertheless, when the six African-American patients were excluded, the measures of associations, i.e., odds ratios, grew stronger, i.e., higher, although the confidence intervals appeared wider due to the smaller sample size. Recall bias, though usually of concern, is not likely to have influenced these results because no associations were seen with asbestos or silica.

Overall, the 69% of cases were 3.75 times as likely to be exposed to at least one of the pertinent exposures; 37.5% of the controls had such an exposure. Calculation of an etiologic fraction suggests that these exposures can explain only about 20% of the sarcoidosis burden. The competing infectious disease hypothesis remains to be tested formally.

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IMMUNOCHEMICAL DETECTION OF MYCOTOXINS ASSOCIATED WITH STACHYBOTRYOTOXICOSIS

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ABSTRACT:

Using high-affinity monoclonal antibodies (Mab) against roridin A which exhibit cross-reactions with satratoxins and other macrocyclic trichothecenes several immunochemical methods were developed for the direct detection of satratoxins in contaminated building materials. Applying a lab-independent enzyme-linked immunofiltration assay (ELIFA) technique positive results were obtained within 10 min for toxin concentrations of > 200 µg/kg. The detection limit of a microtiter plate assay format was 1 µg/kg. For samples contaminated with *Stachybotrys chartarum*, an excellent agreement could be observed between the enzyme immunoassay (EIA) and a cytotoxicity test. The results of the EIA could be confirmed by HPLC analyses using immunoaffinity columns for sample clean-up. Furthermore, a new method was established for the sensitive detection of verrucarol in serum. In three out of 58 serum samples traces of verrucarol were detected. Two other sera reacted strongly positive for macrocyclic trichothecenes. Altogether, these results underline the importance and usefulness of immunochemical methods for epidemiological studies on airborne mycotoxins in indoor environments.

INTRODUCTION

Since the discovery of aflatoxins in the early 1960s, importance of mycotoxin analysis for food and feed hygiene grew steadily over the years due to the high toxicity of these low molecular weight compounds. To minimize the risk of human exposure to aflatoxins, rigorous programs have been established for monitoring these toxins in food. Consequently, analytical methods which are sensitive, rapid, easy to perform and reliable were required. This situation led to the development of immunochemical methods which fulfill these criteria in an excellent way, since they can be designed as lab independent field tests or classical enzyme immunoassays performed in microtiter plates. Additionally, antibodies can be used for the preparation of immunoaffinity (IA) columns, which strongly improve cleanup procedures for physicochemi-

cal detection methods. During the last decade a broad panel of polyclonal and monoclonal antibodies against mycotoxins, i.e. aflatoxins, ochratoxins, citrinin, fumonisins, group A and B trichothecenes, macrocyclic trichothecenes, has been developed in our and other labs (for a review see Chu, 1996).

Immunochemical methods are widely accepted as screening methods in the field of food and feed hygiene documented by the fact that several immunoassay protocols have been adopted as first action methods by the AOAC International. During the last years, besides health hazards due to ingestion of food contaminated with mycotoxins, the inhalation exposure risk has attracted scientific and public attention (Hendry & Cole, 1993; Hintikka & Nikulin, 1998). Particularly, a report published by Croft et al. (1986) describing an apparent case of stachybotrys-toxicosis in people living in a water-damaged building heavily infested with *Stachybotrys (S.) chartarum* stimulated work on improved microbiological diagnosis and air sampling methods. Though several studies were published implying a cause-effect relationship between airborne mycotoxins and disease (Etzel et al. 1998; Hodgson et al. 1998; Johanning et al. 1996), progress in this special area of mycotoxin analysis was rare. This paper describes the application of immunochemical methods for the detection of satratoxins, both in building materials contaminated with *S. chartarum*, and in sera of exposed humans.

General remarks on immmunochemical methods for mycotoxins

Immunochemical methods are based on the ability of antibodies to bind specifically to different substances. In contrast to conventional analysis, enzyme immunoassays do not require sophisticated equipment, but do require certain reagents, namely, specific antibodies and (enzyme) labeled antigens. As mycotoxins are low molecular substances, they are not intrinsically immunogenic. These so-called haptens are made immunogenic by covalent linkage to a suitable macro-molecule, usually proteins such as bovine serum albumin. A number of reviews discussing in detail strategies for the production of antibodies against low molecular substances have been published. (Chu, 1996; Märtlbauer et al. 1994; Stanker & Beier, 1996).

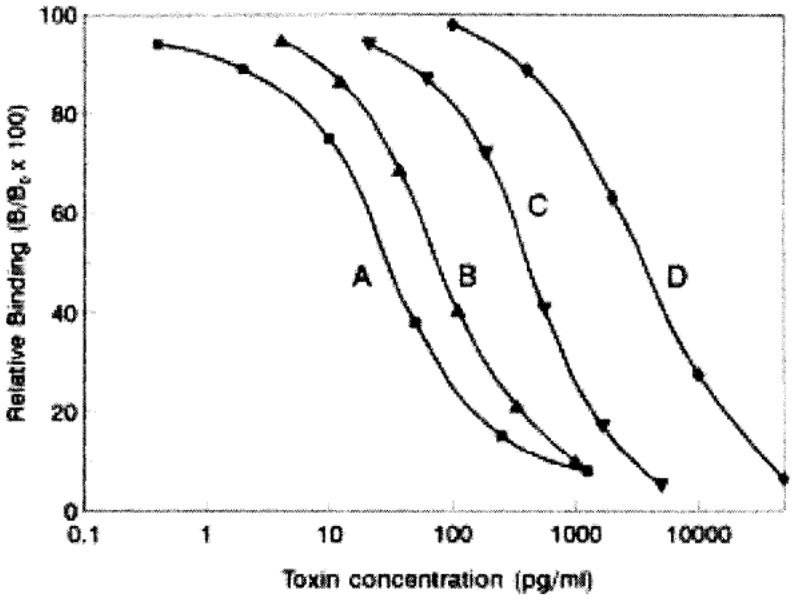
As antibodies are the central component of any immunoassay, their quality mainly determines sensitivity and specificity of the test system. Since haptens such as mycotoxins possess only one antibody binding site, the competitive assay format is mandatory. The competitive method is based on the competition of free and labeled antigen for a limited number of antibody binding sites. In most cases the assay response represents the bound labeled antigen, which is inversely proportional to the concentration of the analyte. One of the most popular assay formats is the enzyme-linked immunosorbent assay (ELISA) and horseradish peroxidase (HRP) often serves as the enzyme label due to its high specific activity. Some examples of competitive EIAs for mycotoxins developed in our laboratory, using either polyclonal or monoclonal antibodies are shown in Table I.

TABLE I: Detection limits (ng per ml; determined by Student's test with a confidence level of 95%) of competitive assays by using polyclonal (Pab) and monoclonal (Mab) antibodies against mycotoxins.

Target compound	Pab	Mab	Reference
Aflatoxin B1	0.01	0.1	6
Aflatoxin M1	0.006	0.05	6, 18
Ochratoxin A	0.05	0.2	6, 19
Citrinin	0.8	0.07	1, unpublished
Roquefortine C	0.8	--	unpublished
Fumonisin B1	0.1	0.2	26, unpublished
Zearalenone	0.07	0.2	6, 27
T-2 toxin	0.21	0.01	9
Diacetoxyscirpenol	0.02	30	17
3-Acetyldeoxynivalenol	0.2	2.2	6, 28
15-Acetyldeoxynivalenol	0.4	4.3	28, unpublished
Deoxynivalenol	8	15	unpublished
Nivalenol	55	--	unpublished
Roridin A	0.7	0.1	10, 20
Verrucarin A	0.2	--	unpublished

Besides sensitivity, another important assay parameter is the specificity of the antibodies employed. Cross-reactivity of antibodies with compounds that closely resemble the target analyte is mainly determined by the choice of the coupling site. In many cases, the part of the molecule that is opposite to the coupling site acts as the immunodominant portion of the hapten-protein conjugate. Structural changes in the vicinity of the coupling site will show less influence on the immmunoreactivity. For instance, for the production of monoclonal antibodies (Mab) against roridin A the immunogen was prepared by coupling the toxin via the macrocyclic ring system to the carrier protein. Thus, the trichothecene skeleton and the parts of the macrocyclic ring system close to this backbone represent the immunodominant structure for this antibody and therefore the Mab showed cross-reactions (Figure I) with a broad range of other macrocyclic trichothecenes such as satratoxin H.

FIGURE I:. Standard curves for some mycotoxins (A, diacetoxyscirpenol; B, diacetylverrucarol; C, roridin A; D, satratoxin H) in competitive enzyme immunoassays using rabbit antiserum against diacetoxyscirpenol (A,B) and monoclonal antibodies against roridin A (C,D), respectively.



IMMUNOCHEMICAL ANALYSES OF BUILDING MATERIALS FOR MACROCYCLIC TRICHOTHECENES

Air sampling methods for *S. chartarum* are prone to false negative results due to the sticky nature of the spores which reduces the likelihood of their dispersion as an aerosol. Therefore assessment of *S. chartarum* in indoor environments relies on the isolation of the fungi from contaminated materials. However, during the last years it turned out that 30 - 50% of all *Stachybotrys* strains have to be considered as weakly toxic or even atoxic since they are not able to produce satratoxins or other cytotoxic components. That means that in addition to mycological assessment mycotoxin analyses, particularly for satratoxins, are required to correctly diagnose an outbreak of stachybotryotoxicosis. However, evaluation of toxin production of fungi grown under laboratory conditions on synthetic media is of limited value, since the level of mycotoxin production by a toxigenic fungus depends very much on the specific isolate and the growth conditions (Jarvis, 1995). As the direct analysis of bulk samples contaminated with *Stachybotrys* is an analytical challenge for classical physico-chemical methods, there is a need for alternative screening methods. Among the bioassays, which generally lack specificity, the MTT-test has been proven its worth as a versatile tool for the screening of mold-infected building materials (Gareis, 1995). However, for the evaluation of the cytotoxicological data obtained, appropriate control samples are required, which are not always available. The aim of the study was therefore to demonstrate the applicability of the EIA for the analysis of macrocyclic trichothecenes in building materials contaminated with *Stachybotrys*, and to check the agreement between the results obtained by EIA and the MTT-test.

Comparison of EIA in microtiter plate format and MTT-test.

For this purpose, field samples, e.g. wall paper, insulation material, ceiling tiles, gypsum paper, were collected in problem buildings, extracted with methanol and analyzed by the MTT-test as described previously (Gareis, 1995). An aliqoute of the extract was evaporated to dryness, redissolved with phosphate buffered saline (PBS) containing 10% (v/v) methanol and analyzed by a competitive EIA for roridin A. Briefly, microtiter plates were coated overnight at room temperature with anti-mouse immunoglobulines (10 µg/ml). Free binding sites were blocked for 30 min with PBS containing 3 % (v/v) fetal calf serum.Then, a buffer solution containing monoclonal antibodies against roridin A (Mab 5G11; 16 ng/ml; 100 µl per well) was added and incubated for 1 h. The plate was washed and verrucarin A-HRP (VA-HRP; 1:20,000; 50 µl per well) was added together with 50 µl of roridin A standard solutions or 50 µl of sample extract solution and incubated for 1 h at room temperature. The plate was washed again, and enzyme substrate solution was added (100 µl per well). After 20 min the enzyme reaction was stopped, and color development was measured. The absorbance data were evaluated using a competitive EIA calculation software. Under these conditions the detection limit of the EIA was 1 ng roridin A-equivalents per gram sample.

Table II shows some of the results obtained with the EIA and the MTT-test. All control samples were negative in the EIA thus demonstrating another advantage of immunochemical methods: laborious and time-consuming extract clean-up procedures can be simplified or even totally omitted. An excellent agreement between the EIA results and the MTT-test could be observed for those samples which were contaminated with toxic strains of *Stachybotrys atra*. Maximum toxin concentrations as determined by EIA were in µg/kg range (roridin A equivalents). Considering that the cross-reactivity of the antibody with satratoxin H is approximately 15%, the actual toxin concentrations in these samples were estimated to be in the range of 0.05 to 15 mg/kg.

TABLE II: Analysis of contaminated building materials by enzyme immunoassay (EIA) and cytotoxicity test (MTT-test). *Stachybotrys* contamination in the samples was qualitatively determined by electron microscopy. For quantitative evaluation of the EIA data a roridin A standard curve was used.

Sample[1]	S. chartarum	**Cytotoxicity**	**EIA (ng roridin A equivalents per g)**
Wall paper	+	+	7
Wooden ceiling	+	+	10
Wall paper	+	+ + +	28
Insulation material	+ +	+ +	31
Gypsum board	+ + +	+ + +	70
Wall paper	+ + + +	+ + + +	2500
Insulation material	+ + + (+)	+ + + + (+)	317 - 1164

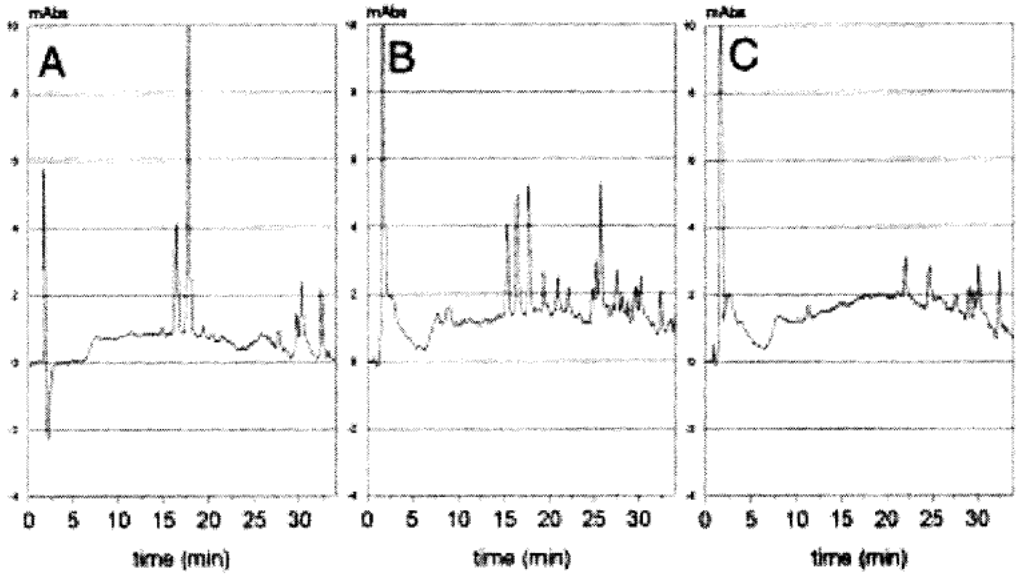
[1] All control samples were negative in the EIA.

Immunoaffinity chromatography.

To further verify the results obtained by EIA, some of the samples also were analyzed by reversed-phase (C18) HPLC with a photodiode array detector. Since HPLC analysis of bulk

sample extracts purified by convential procedures, is often hampered by matrix peak interferences in the chromatograms, an immunoaffinity chromatography (IAC) clean-up method was developed. For this purpose, Mab 5G11 (15 mg) was covalently coupled to 1 g CNBr-activated Sepharose 4B according to the instructions of the manufacturer (Pharmacia, Freiburg, Germany). For preparation of IA columns, portions (100 µl) of the gel were filled into disposable minicolumns (MobiTec, Göttingen, Germany). The toxin binding capacity of the immunosorbens was tested by applying supersaturing amounts of roridin A to the columns. Bound toxin was eluded with 2 ml methanol, and the toxin concentration was measured by EIA. The capacity of this immunosorbens was found to be 18 µg of antigen per ml of gel, which corresponds to a calculated remaining specific activity of the antibody of 51%. In order to check the applicability of the IAC columns, sample extracts (diluted with PBS) from contaminated building materials were applied to the columns. Complete binding of the antigens to the immobilized Mabs was verified by analyzing the extracts passed through the IA column by EIA. Since in IA columns the antigen-antibody reaction is performed under non-competitive conditions, cross-reactive toxins also will be bound to a high percentage of >90%. HPLC analyses of the corresponding eluates (Figure II) indicated, that toxic sample extracts were contaminated both with satratoxin H (RT 17.8 min) and other macrocyclic trichothecenes (RT 15.4, 16.4, 19.8 min), which had typical UV-spectra but could not be further identified due to the lack of appropriate toxin standards. Jarvis et al. (1998) reported about isolates of *S. chartarum*, which produced under laboratory conditions a number of highly toxic compounds, e.g. satratoxin G, isosatratoxin G, and isosatratoxin F. The excellent clean-up provided by the IAC

FIGURE II:. HPLC chromatograms of (A) satratoxin H standard solution, (B) insulation material contaminated with *S. chartarum*, and (C) control sample extract after clean-up with immunoaffinity chromatography. UV-absorbance was monitored at 260 nm, full spectra were taken from 200 - 300 nm. The retention time of satratoxin H was 17.8 minutes.



columns could be demonstrated by the chromatograms of purified sample extracts from uncontaminated building materials, for which no matrix peaks at the retention time window of the macrocyclic trichothecenes were observed (Figure II).

Enzyme-linked immunofiltration assay (ELIFA).

On-site testing for macrocyclic trichothecenes would provide the industrial hygienist and the consulted physician with rapid and very helpful informations about potential health hazards and remediation management. Since enzyme immunoassays can be designed as lab-independent detection methods, and due to the promising experiences with the EIA system described above, it was obvious to develop an immunochemical test system which could be used for on-site testing. Based on previously described studies on lab-independent detection systems for fumonisins and paralytic shellfish poisoning toxins (Schneider et al. 1995; Usleber et al. 1995) we focussed on the development of an enzyme-linked immunofiltration assay (ELIFA). The test device used consisted of a nylon membrane coated with anti-roridin A antibodies (Mab 5G11), which is tightly pressed to an absorbent cellulose layer. Sample extract solution, enzyme conjugate (VA-HRP) solution and enzyme substrate-chromogen solution were sequentially added onto the membrane. The test can be evaluated visually by comparing the intensity of the resulting colored dot with that of a negative control. Qualitative results could be obtained within ten minutes. This ELIFA was used for a rapid presence/absence analysis of toxins in sample extracts of contaminated building materials (Table II). Preliminary results showed that it is possible to detect macrocyclic trichothecenes in these samples at levels above 200 µg/kg. Further studies are aimed at improving the extraction step, which would enable the development of a completely self-contained testing device.

Immunochemical detection of Stachybotrys chartarum toxins in sera

Since the results described above clearly demonstrated that immunochemical assays based on Mab 5G11 represent sensitive analytical tools for the detection of macrocyclic trichothecenes produced by *S. chartarum*, this technique was also used for the analysis of sera of exposed people. First experiments showed, that a simple dilution (1:3 in PBS) of the sera was sufficient to minimize nonspecific interferences in the microtiter plate assay format. However, analysis of 58 serum samples in a pilot study (single blind protocol) indicated that it was not possible to differentiate between sera of exposed people and controls. There are several conceivable explanations for these results: (i) most of the sera were taken some weeks or even months after the actual exposure to *Stachybotrys* (ii) metabolism of the toxins, which may result in major structural changes of the molecule reducing or even eliminating reactivity of the antibodies with these metabolites. However, though the metabolism of type A trichothecenes, e.g. T-2 toxin and diacetoxyscirpenol (DAS), was extensively studied, there are only very few reports about the fate of macrocyclic trichothecenes. In vitro studies showed that one metabolite commonly found is verrucarol (Gareis, unpublished results), which represents the basic structure of all macrocyclic trichothecenes after hydrolysis of the ring linking at C-4 and C-15 in methanolic sodium hydroxide. The Mab 5G11 employed in this study, however, shows no cross-reactions with verrucarol.

In this situation, there were several possible strategies for the specific detection of verrucarol in sera by enzyme immunoassays: (i) production of specific anti-verrucarol antibodies, (ii)

acetylation of verrucarol with acetic anhydride and analysis of the resulting diacetylverrucarol using cross-reactive antibodies. Since experiences with other polar trichothecenes such as deoxynivalenol and nivalenol, suggested that the former approach was likely to be difficult and time-consuming, we focussed on the latter, which took advantage of the availability of an anti-DAS antiserum (Klaffer et al. 1990) having strong cross-reactivity with diacetylverrucarol (Figure I). Additionally, acetylation of trichothecenes possessing hydroxyl-groups is a straightforward and reproducible reaction procedure, which was originally described by Chu et al. (1989) for the sensitive immunochemical detection of polar trichothecenes. The fact that the polyclonal antibodies used for the detection of diacetylverrucarol also react with diacetoxyscirpenol did not hamper the analyses, since the natural occurrence of this mycotoxin is negligible and more important the half-life of DAS *in vivo* is less than 1 h (Bauer et al. 1995).

The conditions required for direct acetylation of verrucarol (without clean-up) in serum were established by analyzing artificially contaminated samples. The acetylation procedure was performed as described by Schmitt et al. (1996). In a concentration range of 5 to 20 ng/ml, the mean recovery was 133 % (CV 10.5), which represents a reasonable result taking into account that verrucarol standard was used for the spiking and acetylation procedure, while a diacetylverrucarol standard curve was used for the evaluation of the absorbance data. The lowest detectable amount of verrucarol, calculated from the limit of detection of the EIA and the sample dilution factor (200) was at approximately 2 ng/ml. In a modified test procedure involving liquid-liquid partition as a clean-up step prior acetylation the limit of detection for verrucarol in serum was at 0.5 ng/ml. This procedure was more time-consuming, but could be used for confirmation.

In a second study, again designed as single blind protocol, 60 sera and one known control were analyzed both for macrocyclic trichothecenes and verrucarol. In the diacetylverrucarol EIA, three samples gave a weakly positive result causing an inhibition binding of the DAS-HRP employed in the EIA of 25%. For comparison, analysis of sera artificially contaminated with 5 ng verrucarol per ml resulted in an inhibition of approximately 45%, whereas the control serum had no inhibitory effect on the binding of the DAS-HRP (inhibition < 5%). After clean-up of the samples with liquid-liquid partition the three sera still reacted positive albeit to a less extent. Furthermore, two other sera reacted strongly positive in the EIA for macrocyclic trichothecenes (inhibition 38 and 45 %). Assuming that only satratoxin H was present in these sera this response would correspond to a toxin concentration of 5 - 7 ng per ml. Interestingly, it turned out that the two sera were from the same outbreak taken during actual exposure, whereas the serum samples which contained traces of verrucarol were collected several weeks post exposure. To our best knowledge this is the first report about the detection of macrocyclic trichothecenes and their metabolites in sera of people exposed to *S. chartarum*. However, again most of the analyzed sera of exposed people reacted negative in both EIAs, and therefore there is only limited evidence that mycotoxins represent reliable biomarkers in serum, particularly because the half-life of satratoxins is probably only a few hours. Additionally, in most cases individuals present for medical evaluations long after their acute *Stachybotrys* exposure. Nevertheless, positive EIA results give strong evidence for recent exposure to *S. chartarum* toxins thus emphasizing the importance of such blood analyses. The impact of these findings for environmental medicine is significant and requires more intensive research. Further work will

aim in the improvement of the verrucarol assay with respect both to sensitivity and performance.

CONCLUSIONS

The potential of the immunochemical approach for the detection of mycotoxins in indoor air environments could be clearly demonstrated. Designed as lab independent field tests or classical microtiter plate EIAs, immunochemical methods enable the direct analysis of contaminated building materials and are suitable for the sensitive detection of mycotoxins in sera, thus providing essential data for a profound risk assessment. To further clarify the role of mycotoxins in the pathogenesis and pathophysiology of building-related illnesses, production of specific antibodies against mycotoxins of other fungi often found in water-damaged buildings would be desirable.

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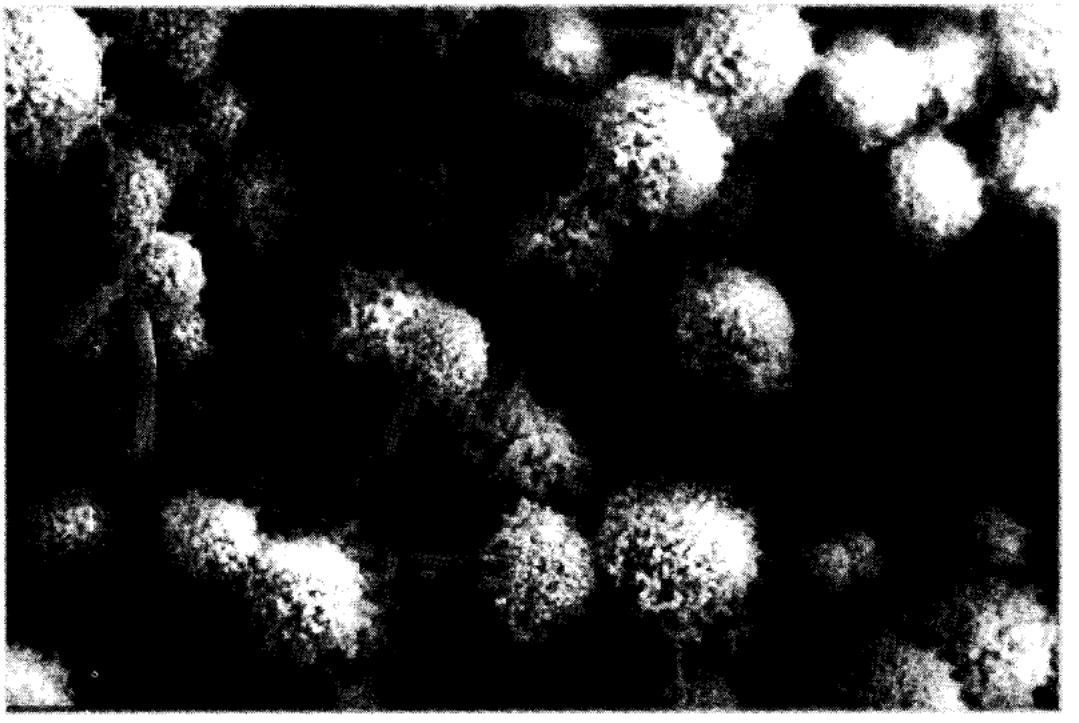

Aspergillus heads, EM 580 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands.

Chapter 5

Prevention

Mitigation

Control

MITIGATION OF VISIBLE FUNGAL CONTAMINATION IN BUILDINGS: EXPERIENCE FROM 1993 - 1998

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ABSTRACT:

Experience in the early 1990's led to the recommendation that removal of visible fungal growth from interior surfaces in buildings be performed in a manner that minimized the dispersion of particles (dusts) in indoor air. Several documents beginning with the 1994 New York City Guidelines on Assessment and Remediation of *Stachybotrys atra* in Indoor Environments provided practitioners with procedures that can be used to remove visible fungal growth from building interiors. All fungal remediation guidelines recommend that sustained and extensive fungal growth on interior surfaces should be physically removed and that people performing remediation work should use appropriate personal protective equipment. In addition, all guidelines published in 1993-1998 recommend that moisture problems in building infrastructure be fixed in order to prevent new fungal growth. Fungal remediation guidelines specify a certain surface area of visible fungal growth (generally 3 to 10m^2) that requires containment barriers similar to those used when hazardous chemical or physical materials are removed from buildings. Misunderstanding of 1993-1998 guidelines has resulted in both overly conservative approaches to clean-up as well as to dispersion of fungal spores throughout a building because of poor dust control. Unlike guidelines on removal of hazardous chemical and physical agents where rigid inspection protocols and specific numerical guidelines are appropriate, the removal of mycobiota is a variable process depending on many factors including the biology of the fungal taxa. Factors such as the following should be considered during the remediation process: (a) the location, extent, and kind of fungal growth in building systems, (b) the susceptibility of building materials to biodeterioration, (c) the porosity of building materials, (d) the susceptibility of occupants to bioaerosol exposure, and (e) sampling and monitoring protocols appropriate for the fungal contaminants. Fungal remediation in buildings continues to require a considerable degree of professional judgement with regard to procedures appropriate for containment of dusts and for control of the contaminant mycobiota.

KEY WORDS

Fungi, Remediation, Colonization, Dust, *Stachybotrys chartarum, Aspergillus versicolor*

INTRODUCTION

In 1994 one of us described procedures used in large scale remediations in several Florida buildings where interior surfaces had been massively colonized by fungi (Morey, 1995). In that publication, specifications for large scale fungal remediation were suggested including containment and isolation of areas with visual fungal colonization, use of personal protective equipment by those performing clean-up work, removal of colonized debris and associated dusts, and sampling to demonstrate that the affected building or building component has been returned to "normal" status by remediation and cleaning.

During the period from the First International Conference at Saratoga Springs, New York (Johanning and Yang, 1995) to the present, several conferences and consensus panels have published reports containing recommendations on the subject of remediation of fungal colonization in buildings (Samson et al. 1994; New York City, 1994; Health Canada, 1995; International Society of Indoor Air Quality, 1996; American Conference of Governmental Industrial Hygienists, 1999). Following a review of these reports, the general principles that should be considered during fungal remediation are discussed. Finally pre-remediation and post-remediation visual inspection and sampling data are presented for three buildings that underwent large scale fungal remediation.

REVIEW OF PUBLISHED REPORTS

LEVITICUS (BLOMQUIST, 1994)

Over 30 centuries ago it was recognized that structural materials such as plaster, mortar, and wood which are affected by sustained fungal growth should be physically removed from buildings. It was also recognized that people may be affected by fungal growth at least to the extent that they should wash their clothes after being present in the contaminated building.

INTERNATIONAL WORKSHOP IN BAARN (SAMSON ET AL. 1994)

A number of recommendations as follows were made at the Baarn workshop. The most important recommendation was that from a health and hygiene point of view, fungal colonization of interior surfaces in buildings was unacceptable. It was also recognized that as a prerequisite to clean-up of colonization, building moisture problems must be fixed. Because of the unknown health effects of biocides, their use during clean-up operations should be considered only when other approaches to cleaning are not feasible.

NEW YORK CITY GUIDELINES (1994)

A panel convened in 1993 in New York City recommended remediation (clean-up) procedures to be used when small isolated areas ($\leq$2 square feet [SF]), larger isolated areas (2 SF to approximately 30 SF), and extensive surfaces (>30 SF) are colonized by fungi. The panel was convened because of fungal growth problems (primarily *Stachybotrys spp.*) on cellulosic substrates (e.g., paper fiber gypsum board, books, paper files) in New York City buildings.

An important recommendation of this panel was the complete isolation of the affected area (use of plastic sheeting and negative pressurization) when more than about 30 SF of colonized surface is remediated. The intent of this recommendation was to prevent spores aerosolized during clean-up activities from entering other areas of the building. The New York City Guidelines stated that remediations involving colonization of <30 SF can be performed... "by regular maintenance staff." However, maintenance personnel must participate in a full respiratory protection program. The New York City Guidelines did not recommend the use of containment barriers (e.g., plastic sheeting, negative pressurization) for remediation of colonized areas of <30 SF.

Health Canada Fungal Contamination Guide (1995)

The Health Canada 1995 publication on "Fungal Contamination in Public Buildings: A Guide to Recognition and Management" contains an overview of standard operating procedures to be followed during remediation of materials contaminated by fungi. The use of gloves and respirators are recommended during removal of colonized materials even for small scale (<3 SF) operations. When large scale clean-up operations occur (colonized surface of 100 or more SF), engineering controls such as the use of negative air machines (air filtration devices, AFDs), use of containment barriers, and use of high efficiency particulate air (HEPA) filter vacuum cleaners are required. During large scale fungal remediation, evacuation of occupants elsewhere in the building should be considered.

International Society of Indoor Air Quality Guidelines (1996)

The International Society of Indoor Air Quality (ISIAQ) published a 1996 guideline on "Control of Moisture Problems Affecting Biological Indoor Air Quality" which contains a section on clean-up and decontamination methods. This guideline recommended that soft porous materials colonized by fungi should be discarded (see also CMHC, 1993). The type of clean-up activities recommended in the New York City guidelines (1994) for small isolated areas (< 2 SF), larger isolated areas (2 SF to approximately 30 SF), and extensive areas (>30 SF) colonized by fungi was reaffirmed. The ISIAQ guideline noted that physical removal of colonized fungi is required for all taxa with urgency given if toxigenic species are present. The goal of clean-up activities according to the ISIAQ guideline should be to reduce fungal taxa on surfaces to background levels that would occur normally in a dry non-problem building.

American Conference of Governmental Industrial Hygienists (ACGIH) Bioaerosols Committee (1998)

At the time of writing the draft of the ACGIH 1999 publication on "Bioaerosols: Assessment and Control", reiterates many of the principles of earlier guides (above) such as the need to eliminate moisture problems that allowed colonization to occur and the need to physically remove porous materials with extensive colonization. In a departure from other reviewed guidelines the ACGIH Bioaerosols Committee uses the terms minimal, moderate, and extensive to classify the extent of colonization without assignment of numerical surface area values to these descriptive terms. The terms "source containment, local containment, and full con-

tainment" are used to apply to remediations when minimal, moderate, and extensive colonizations, respectively are removed (ACGIH, 1999).

Plastic sheeting barriers and negative pressurization are recommended to contain dusts when extensive areas of colonization are removed. At a minimum, N-95 respirators should be used by persons performing clean-up regardless of the extent of colonization. Professional judgement should be used when deciding if higher levels of respiratory protection are needed during remediation.

BASIC PRINCIPLES FOR FUNGAL REMEDIATION

Fungal remediation in buildings includes (a) the physical removal of colonization from interior surfaces, (b) prevention of dusts generated during removal of colonized materials from entering occupied or clean areas, (c) removal of settled dusts containing spores that may have previously been dispersed from moldy surfaces, and (d) use of respirators and protective clothing by clean-up workers. A plan to prevent future leaks or dampness that caused colonization is essential for effective remediation.

Porous materials such as paper fiber gypsum board, ceiling tiles, insulation, wall paper, carpet, clothing, etc. that are visually colonized should be discarded. Cellotape sampling is useful to distinguish colonized (hyphae or fruiting structures present on the substrate) from phylloplane spores that normally settle on sound surfaces.

Fungal colonization that may be present on non-porous surfaces such as sheet metal, ceramic tiles, glass, etc., can be physically removed by cleaning. Tap water with detergents should be adequate for most cleanings.

The method used to remove colonization on semi-porous materials such as wood depends on the degree to which hyphae have penetrated into wood cells. Wood that is dry rotted or wet rotted and unsound (Lloyd and Singh, 1994) is discarded. Wood that is sound with the exception of colonization of the outer layers of wood cells (approximately 1 mm depth) may be sanded, planed, refinished, and reused. The general principle for reuse should be absence of hyphae and fruiting structures (above that normally present in sound timber) in the wood cells of the timber being salvaged.

The airborne concentration of spores can exceed $10^6/m^3$ when moldy materials are removed during clean-up (Rautiala et al. 1996, Morey and Ansari, 1996). Consequently those persons involved in clean-up activities must use respirators and protective clothing. The use of a N-95 respirator and gloves is adequate during the clean-up of areas where the extent of colonization is minimal (ACGIH, 1999). For remediations involving removal of extensive colonization the use of full body disposable protective clothing and respirators (P-100 or HEPA cartridge) must be considered.

The use of dust suppression techniques and/or containment barriers during removal of moldy materials is essential to prevent dissemination of spores into occupied or clean areas. The extent of the surface colonized (minimal, moderate, extensive) in a room or in one area of a building is the most important factor to be considered with regard to use of dust suppression techniques and/or containment barriers (Table I). Other factors important in determining dust

containment procedures employed during a remediation include (a) the presence of highly susceptible occupants (Streifel, 1988) and (b) the likelihood that hidden colonization may be uncovered within building components (For example, within building envelope or flooring systems).

When extensive visible fungal growth is removed from a room or from one area of a building, the moldy surfaces are isolated from the rest of the building and the HVAC system by use of plastic sheeting (containment barriers). The isolated area is depressurized through use of engineering controls such as a negative air machine(s) which discharges spore laden air outdoors through HEPA filters (spores trapped in filter). The flow of conditioned air in the building is always from clean areas into the rooms undergoing remediation.

A principle common to guidelines on fungal remediation (NYC, 1994; ISIAQ, 1996) is that building maintenance people can perform remediations involving minimal and moderate surface area colonization. For example where 10 to 20 SF (1 to 2 m^2) of visible fungal colonization occurs on paper fiber gypsum board, this contamination can be removed in a mini-enclosure. The colonized area is isolated from room air by a plastic sheet and negatively pressurized by insertion of the nozzle of a HEPA vacuum into the enclosure. The clean-up person with appropriate personal protective equipment (full body disposable suit, gloves, full face respirator with N-95 or better cartridge is recommended) (a) enters the enclosure, (b) removes and bags moldy materials, and (c) prior to exiting the enclosure uses the nozzle of a second HEPA vacuum cleaner to remove settled dust from the outer surface of the bagged trash, from the surfaces of sound finishing material that is being retained, from the inner surface of the plastic sheeting, and from the surfaces of disposable protective clothing and equipment.

Porous materials with surfaces colonized by minimal fungal growth (e.g., 1 or 2 ceiling tiles, 1 or 2 SF [0.1 or 0.2 m^2] of paper fiber gypsum board) can be removed by persons wearing gloves and a respirator. As a means of controlling dust generation, items such as moldy ceiling tiles can be carefully placed and sealed in a plastic bag and then removed. Also, the colonized paper surface of gypsum board can be covered with a sticky sheeting (sticky surface makes contact with colonized surface), extracted from the wall in one piece, bagged, and removed.

During removal of moldy finishing and construction materials, any technique that reduces dust emission from the colonized surface should be considered. Thus, application of an encapsulant to a colonized surface or simply damp wiping of the surface may suppress dust (spore) emission. The application of a gentle mist to colonized surfaces may be effective in dust suppression if hydrophobic spores are not dispersed into the air by impaction of water droplets. However, water mists and sprays, if used, must not wet sound infrastructure.

Spores from colonized surfaces may have been previously dispersed long distances on air currents into areas of the building not directly affected by moisture problems (Morey, 1995). A combination of damp wiping (non-porous surfaces) and HEPA vacuum cleaning should be adequate to remove most of the dusts containing settled spores.

The objectives of remediation are to physically remove colonization and associated mold laden dusts, but not to sterilize or disinfect interior surfaces. Since physical removal of colonization and moldy dusts is the objective of remediation, the use of biocides and disinfectants to kill spores is unnecessary.

GENERAL CONSIDERATIONS AND DEFINITIONS

The process of remediation of fungal colonization in buildings begins with an inspection of the building and its infrastructure. The location and nature of water damaged materials as well as the source of moisture problems must be understood and fixed if the restoration is to be permanent. Determining the location and extent of visible fungal colonization (minimal, moderate, or extensive) is the most important factor in the evaluation process leading to fungal remediation (Table I). Based on the extent of surface area colonization, the remediation process may be described as source removal, local removal, or a full containment removal (ACGIH, 1999; Morey, 1996; Table I) as follows:

Source Removal

This type of removal is used when minimal (less than 4 SF) visual growth (colonization) of fungi occurs on interior surfaces. Plastic sheeting barriers are not required. However, common sense and inventiveness are required by persons removing moldy materials, especially with regard to minimizing or preventing dispersion of spores from moldy surfaces. Thus, the colonized portion of a wallboard surface may be entirely covered by polyethylene sheeting (tightly secured at the edges with duct tape) and the covered portion extracted (cut out) in one piece, sealed in a disposable bag, and removed from the building. Alternatively, a sticky sheeting or an encapsulant may be applied to the entire colonized surface in order to minimize potential aerosolization of spores prior to extraction from the wall. The nozzle of an operating HEPA vacuum cleaner is used to capture dusts aerosolized when moldy materials are removed from building components. When mold growth occurs on non-porous materials such as diffusers, drain pans, or metal components of fan coil units, local use of disinfectants and tap water is acceptable for cleaning purposes. At a minimum, workers performing source removal of minimal amounts of fungal growth must utilize a N-95 respirator and disposable gloves.

Local Removal

This type of removal is used when moderate (4 SF to 30 SF) visual colonization occurs on interior surfaces. A single or double layer of plastic sheeting (mini-enclosure) may be used to isolate work areas where colonized materials are being removed. This plastic sheeting is used to keep dusts and spores from entering spaces outside the clean-up areas. One or more large HEPA vacuum cleaners (canister outside plastic sheeting; nozzle inside enclosure) are used to negatively pressurize the work area inside the mini-enclosure. Professional judgement is required with regard to the dust containment methods used for removal of moderate amounts of visual colonization. Thus, depending upon the building and the nature of the colonization, other local removal methods in addition to the mini-enclosure may be used during remediation. For example, several moldy ceiling tiles can easily and simply be removed from a room as described under source removal without construction of plastic barriers and negative pressurization systems.

Removal of 10 or 20 SF of moldy plywood from a wall in a room may be accomplished with a mini-enclosure combined with dust suppression methods to keep spores from dispersing into areas outside of the mini-enclosure. Alternatively, a sticky tape or sticky sheeting may be

applied to the 10 to 20 SF moldy surface and the colonized material may be cut out and removed without the necessity of a mini-enclosure.

Full Containment Removal (Large Scale Clean-up)

This type of containment is used when extensive (greater than 30 to 100 square feet) of visual colonization occurs on interior surfaces. Double layered polyethylene containment barriers are recommended to isolate the work area where colonized materials are being removed. These double layered barriers should keep dusts and spores from entering adjacent occupied areas, HVAC system components, and any other building spaces (e.g., return air plenum, risers, wall cavities, etc.). AFDs (negative air machines) are used to negatively pressurize the remediation work area from zones outside of containment barriers. Sound materials that are to be salvaged such as wall board within the confines of the containment should be covered by polyethylene sheeting (protective barriers) depending upon the professional judgement of the on-site environmental hygienist. Full containments should provide maximum isolation of the remediation work area from areas outside containment barriers. The hygienist overseeing the full containment removal process should determine if a single or a tri-chamber decontamination unit is needed and whether clean-up persons should use powered air purifying respirators and one or two layers of full body disposable protective clothing.

Professional Judgement

Unlike guidelines on removal of hazardous chemical and physical agents where rigid inspection protocols and clearance sampling with predetermined numerical guidelines are appropriate, the removal of mycobiota is a variable process depending on many factors including the potential growth of the organisms that may be present. The extent (minimal, moderate, extensive) and location of visible fungal colonization must be clearly understood in order to carry out building restoration. Knowing the extent and location of visible colonization allows choices to be made on the nature of the remediation process, in other words source, local, or full containment removal. Other factors that should influence the nature of the remediation process include the location of highly biodegradeable building materials, the porosity of colonized building materials, and the location of occupants including those with special susceptibilities.

SAMPLING AND FUNGAL REMEDIATION

Consensus guidelines on fungal remediation have recognized that inspection of building systems for the presence of visible fungal colonization and for moisture damage is the cornerstone of the restoration process (NYC, 1994; Health Canada, 1995; ISIAQ, 1996). The extent of visual colonization of interior surfaces is the key factor which categorizes contamination as minimal, moderate, or extensive (ACGIH, 1998; Morey, 1999). This categorization then determines if source, local, or full containment removal (Table I) is applicable in the remediation.

TABLE I: Guidelines for Clean-up of Fungal Colonization on Surfaces in Buildings*.

Visible Fungal Colonization	Type of Remediation	Kinds of Clean-up Activities (see text)
Minimal	Source removal	Remove contaminated materials with minimum dispersal of spores; Use N-95 respirator
Moderate	Local removal	Mini-enclosure and HEPA vacuum cleaner may be used; use N-95 respirator or better; use full body protective clothing
Extensive	Full containment removal	Containment barriers and negative pressurization by AFD recommend ed; use P-100 respirator and full body protective clothing

* Portions adapted from Morey, 1996; ISIAQ, 1996, ACGIH, 1999

Although inspection of building systems for moisture damage and for evidence of visible fungal growth is of primary importance, sampling can have a useful role in the restoration process. Identification of the kinds of taxa on surfaces can be a diagnostic tool in distinguishing if the building moisture problem is associated with chronic dampness (e.g., xerotolerant fungal species should dominate) or chronic flooding (e.g., hydrophilic fungal species should dominate) conditions (ISIAQ, 1996). In addition, culture or direct-microscope analysis of settled dusts at locations remote from water damaged areas can establish if fungal spores from colonized surfaces have been dispersed throughout the building (Morey, 1995). Thus, the dominating presence of non-phylloplane species such as *Stachybotrys chartarum* or *Aspergillus versicolor* in settled dusts in locations remote from colonized surfaces indicates the need for thorough dust cleaning (HEPA vacuuming) which would then become an important part of the restoration process (Morey, 1995).

Sampling during the fungal remediation can also be useful in documenting (a) that dust suppression or control methods used during removal of contaminated materials are effective and that (b) the building has been returned to a mycological "normal" status after the restoration is complete. Several examples where sampling was useful in the restoration process follow:

Building A (Table II)

Prior to remediation extensive visible colonization (>5,000 SF) occurred on paper fiber gypsum board, painted concrete block surfaces, ceiling tiles, the paper surface of chilled water pipe insulation, fire insulation, etc. Air (Table II) and settled dust sampling showed that 90% or more of the taxa present were *Penicillium* or *Aspergillus* species. All visibly colonized materials and visible settled dusts were removed from the building. In addition to a thorough inspection process for removal of all visual colonization and all visible dust, sampling after remediation demonstrated that airborne fungi in indoor air were qualitatively similar in rank order to those present in outdoor air (Table II). Thus *Penicillium-Aspergillus* spores were no longer the dominant indoor taxa.

TABLE II: Airborne Fungal Spores Before and After Full Containment Removal of Extensive Colonization in Building A*.

Time of Sampling	Average Concentration of Ranked Taxa (spores/m³)
Before remediation (N=11)T	*Penicillium-Aspergillus* (7,800) *Cladosporium* (80) *Trichoderma* (60)
After remediation (N=10) T	Basidiospores (1,050) *Penicillium-Aspergillus* (400) *Cladosporium* (100) Ascospores (70)

* Samples collected under quiescent conditions with spore trap operating at a flow rate of 0.01 m³/minute. Basidiospores dominated outdoor air after remediation and basidiospores and *Cladosporium* dominated outdoor air prior to remediation.
T Extensive (>5,000 SF) visible fungal colonization on surfaces before remediation. Visible colonization absent after remediation.

BUILDING B (TABLE III)

Extensive (>100 SF) visible colonization (mostly *Stachybotrys, Chaetomium*, and *Aspergillus* spp.) was present on paper fiber gypsum board of a wall in a room that had been chronically damp or wet for several years. The colonization was remediated in a full containment negatively pressurized enclosure by removing the affected wall board and by HEPA vacuum cleaning of all surfaces within the enclosure.

TABLE III: Simultaneous Collection of Airborne Spores Inside and Outside of a Full Containment During Removal of Extensive Colonization in Building B*.

Location of Sample	Concentration of Ranked Taxa (spores/m³)
Within full containment during removal of moldy wallboard T	*Penicillium/Aspergillus* (90,000) *Stachybotrys* (80,000) *Chaetomium* (30,000) *Cladosporium* (10,000) hyphal fragments (8,000) *Alternaria* (5,000)
Immediately outside of full containment barrier TT	*Penicillium/Aspergillus* (700) *Cladosporium* (700) *Chaetomium* (700) *Stachybotrys* (400)

* Samples collected with spore trap operating at a flow rate of 0.01 m³/minute.
T Extensive (>100 SF) visible fungal colonization present on one wall of a room. Sample collected inside full containment during remediation.
TT Sample collected outside containment in the room approximately six feet away from the plastic sheeting during remediation.

During the remediation an air sample was collected by spore trap within the enclosure and also in the room outside the containment barrier. Concentrations of *Penicillium-Aspergillus*, *Stachybotrys*, and *Chaetomium* spp. within the full containment were about two orders of magnitude higher than those found in the sample collected immediately outside the plastic sheeting (Table III). Air sampling showed that the full containment enclosure used in Building B was effective in keeping most of the spores dislodged within the enclosure form entering areas external to the plastic sheeting. Rautiala et al. (1998) have also found that use of full containment enclosures during remediation is effective in keeping airborne fungi out of adjacent areas. The data in Table III as well as the studies of Rautiala et al. (1998) indicate the need for workers performing remediation to use appropriate personal protective equipment (see also NYC, 1994; Health Canada, 1995; Morey, 1995 for specific recommendations).

Building C (Table IV)

Extensive (>400 SF) visible colonization was present on various surfaces (paper fiber gypsum board, upholstered furnishings, painted surfaces, etc.) in a large instructional room that had been chronically damp. Sampling was performed quiescently in the room before and after full containment remediation. *Aspergillus sydowii, Aspergillus versicolor,* and *Wallemia sebi* were present in air samples collected on DG-18 medium before remediation (Table IV). Only *Aspergillus versicolor* (but not *Aspergillus sydowii* and *Wallemia sebi*) was found in air samples collected after clean-up. Sampling in the same room using malt extract agar, also showed that the concentration of *Penicillium, Aspergillus* and *Eurotium* spp. in the room was similar to the background concentrations of the same taxa in the outdoor air. The remediation in the large instructional room in Building C was judged to be successful primarily because of an absence of visual fungal colonization and because the moisture problem had been fixed. Air sampling was useful because it suggested that airborne fungi in the room were approaching background concentrations of non-phylloplane taxa present in the outdoor air and elsewhere in the building.

TABLE IV: Air Sampling for xerotolerant Fungal Species Before and After Removal of Extensive Colonization in a Large Instructional Room in Building C*.

Time of Sampling	**Average Concentration of Xerotolerant Species (CFU/m³)**
Before remediation (N=7) T	*Aspergillus sydowii* (55 *Aspergillus versicolor* (20) *Wallemia sebi* (8)
After remediation (N=5) T	*Aspergillus versicolor* (11)

* DG-18 culture medium; culture plate impactor operating at 0.18 m³/minute. Average total concentration 300 CFU/m³ before and 190 CFU/m³ after remediation.

T Extensive (>400 SF) visible colonization present on surfaces in room prior to remediation. Visible colonization absent after remediation.

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MICROFUNGAL CONTAMINATION OF DAMP BUILDINGS: BIOLOGICAL ASPECTS

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ABSTRACT

Thie intention with preparing this review has been to present some of the questions and problems connected with moldy buildings, comprising examples of building materials and constructions at risk for mold infestation, identification of the building associated fungal flora - new term: FUNGA - (conf. fauna, flora, funga).

The mode of exposure and some of the fungal components suggested to be involved as some of the etiological agents in adverse health reactions in buildings: allergens, mycotoxins and MVOCs are described and discussed.

Furthermore the nine projects included in "The Danish mold research programme 1998-2001" supported by a grant from the Danish Research Councils are presented. The aim of this multidisciplinary programme is to combat mold growth in existing Danish buildings and to prevent growth in buildings which are going to be constructed.

KEY WORDS:

mold infestation, exposure, mold allergens, mycotoxins, *Penicillium chrysogenum, Aspergillus versicolor, Stachybotrys chartarum, Chaetomium globosum*, sick building syndrome.

MOLD INFESTATION

Mold spores are omnipresent, and a constantly elevated humidity in a given building material containing organic components will inevitably lead to microbial growth and subsequent damage to the materials. Building materials most vulnerable to mold attacks are water damaged, aged organic materials containing cellulose, such as wooden materials, jute, wallpaper and cardboard (Gravesen et al. 1998). Often growth of more than one mold species occur within an area of a material, but only a limited number of species are relevant in connection with the different building materials, named *the building associated funga.*

A mold species can unambiguously be characterized and given a name based on morphology, profile of mycotoxins and other biologically active metabolites in combination with few physiological data (Frisvad, 1998). The experimental data behind species name may predict the potential for growth an production of metabolites under conditions relevant in buildings. Adequate handling of practical problems with moldy buildings such as health implications, extent and mode of renovation and preventive measures depends on knowledge of the above mentioned features.

Mold spores will be introduced to the indoor environment through open windows during summer and autumn, brought in by dirty footwear or simply attached to dust particles. They can grow on e.g. foodstuffs or plants, or they can infest different types of building material. Since molds possess a variety of different very potent enzymes and acids, they can destroy organic materials very efficiently. During mold growth, production and excretion of enzymes may cause the material to deteriorate or even to disintegrate. Besides nutrition, other environmental factors such as pH and temperature play a role for mold infestation, but the key to fungal growth in buildings is moisture. If the mold spores find suitable conditions, which could be different kinds of humid materials, they will grow and damage the material infested.

In a Danish study of water damaged school buildings recently conducted, *rising damp, leaking pipes or water ingress through* roofs were the main reasons for dampness of materials (Gravesen et al. 1999).

Old, dirty, wall-to-wall carpets placed in service buildings such as schools, kindergartens and offices offer likewise an excellent substrate for growth of deposited mold spores, if the carpets are supplied with adequate moisture (Gravesen et al. 1984). Carpet wetting can either be due to accidental water damage, when people walk in with wet shoes or boots, or on purpose. The latter is often the result of cleaning procedures, which use shampoo and water. Prevention of static electricity with humidification of carpets, as is sometimes recommended by authorities may also be the reason for mold infestation.

The assumption that a *relative humidity* (RH) less than 70% will secure no growth of mold is not valid, as the water activity (a_w) of the building materials is the determining factor. In our study of water damaged Danish school buildings *Penicillium chrysogenum, Aspergillus versicolor* (primary colonizers) and *Stachybotrys chartarum* (tertiary colonizer) (Grant et al. 1988) were the three species most frequently isolated (Gravesen et al. 1999).

EXPOSURE TO MOLD COMPONENTS

With few exceptions molds in buildings are non-pathogenic i.e. non-infectious, but possess different *allergenic, toxicological* and *irritative* potencies, owing to their production of biologically very active compounds, which may contribute to adverse health reactions after exposure (Samson et al. 1995, Rylander, 1997, Husman, 1996).

ALLERGENS

In the indoor climate, specific hyperreactivity in genetically predisposed persons is a well described health effect caused by the fungal allergens, which are mainly protein components (Gravesen, 1979, Wanner et al. 1993)

Most filamentous fungi have the ability to produce spores (conidia) in large quantities. The spores are liberated from a growth focus to the ambient air either as single spores or in clusters or chains, or they can be attached to other particles such as dust and textile fibers and sediment on horizontal surfaces. They can be deposited on human skin and in the eyes or be inhaled and extracted from the mucosal surface of the upper airways. Repeated exposure to large amounts of fungal propagules risks development of specific allergic reactions between

certain mold proteins - allergens - and IgE antibodies (Type 1 allergy), or IgG antibodies (Type 3 allergy) respectively. Main symptoms will be hayfever or asthma (Flannigan et al. 1991, Lacey, 1991, Gravesen et al. 1994, Mygind et al. 1996).

The use of allergen extracts to establish the specific diagnosis of mold allergy has been of modest value, as the mold extracts used for in-vivo and in-vitro tests for the specific mold allergies, are not yet of a standardized quality. Besides, extracts of the relevant damp building associated molds are only to a limited extent commercially available.

Mycotoxins

When growing on e.g. damp building material, the molds are able to produce a wide variety of different secondary metabolites e.g. "battle-substances" in order to protect a nutrient source against bacteria or other fungi. Secondary metabolites produced by molds are toxic either to man, animals, plants or other micro-organisms. The toxicological properties and degree of toxicity of these metabolites vary depending on the way they are taken up, the chemical constitution and concentration of the substances produced.

From samples of moldy building materials collected in damp buildings some of the most toxic substances of biological origin such as sterigmatocystin and trichothecenes have been detected, produced by *Aspergillus versicolor* and *Stachybotrys chartarum* respectively (Nielsen et al. 1998, Croft et al. 1986).

A newly conducted study with moistened materials inoculated with six different frequently occurring mold species of the building associated funga demonstrated mycotoxins such as chaetoglobosins, alternariol, 3-methoxy-viridicatin and several others (Nielsen et al. 1999).

Recent research on health implications upon exposure to mycotoxins in damp houses has been concentrated on the respiratory route and the skin contact with the toxic metabolites. Mycotoxins located inside, as well as on the surface of the spores has been demonstrated. This implies that there can be a direct contact to mycotoxins following inhalation of mold spores. Furthermore it is demonstrated that the potency of the respiratory route is higher than the alimentary (Creasia, 1986), which means that the dose of mycotoxin required to cause particular effects is typically one order of magnitude less when inhaled than by ingestion. Some of the trichothecenes are the most potent inhibitors of the protein synthesis, with immunosuppresive and dermatotoxic effects. Breathing 1000 *Stachybotrys* conidia/m^3 air - regardless alive or dead- may give different unwanted effects on the immune system among other systemic effects (Flannigan & Miller, 1994).

Several adverse health reactions of which mucosal- and skin problems and non-specific hyperreactivity of the airways are attributed to exposure of mycotoxins and volatiles (Wanner et al. 1993, Flannigan & Miller, 1994, Husman, 1996). In rare cases symptoms such as nose and lung bleeding have been reported, suggesting exposure to high concentration of *Stachybotrys* spores (Etzel et al. 1998).In comparison with the health effects after exposure to allergens, the effects of mycotoxins and fungal volatiles are incompletely studied and understood, with few exceptions (see Croft et al. 1986, Hodgson et al. 1998 and Etzel et al. 1998).

MICROBIAL VOLATILES (MVOC)

Most odors in damp buildings are caused by mold growth and in some cases by bacteria, especially actinomycetes, but rarely by wood-rotting fungi (Flannigan et al. 1991). Presence of such fungal volatiles may add negatively to the indoor air quality. Mold growth in buildings is often discovered because of the emission of such characteristic musty or earthy odors produced by the molds. One of these heavy musty odors is *2-methyl-isoborneol* produced by several Penicilia and the earthy smelling *geosmin* produced by *Chaetomium globosum*, several *Penicilia*, *Aspergilii* and actionomycetes (Larsen & Frisvad, 1994).

As is the case with the production of mycotoxins, water activity (a_w) may have a considerable influence on volatile production by molds. Experiments with growth of molds on agar have led to the assumption that production of volatiles is inversely related to water activity, and that the volatiles are accumulated during stress periods in which they fulfill an osmoregulatory function after which they can be released (Gravesen et al. 1994).

Due to their deteriorative effect on the indoor air quality microbial volatiles can be very troublesome. Scandinavia and Sweden, in particular, has had considerable mold problems because houses are built directly onto the soil above the underlying rock. The floor is then placed on a concrete slab often without a waterproof barrier between the concrete and the soil resulting in mold attacks on the floors and subsequent contamination of houses. Curtains, carpets and clothing of people from moldy houses will absorb the unpleasant odors, and people coming from such moldy houses very often carry these musty smells around resulting in social isolation or teasing of their children in school. Mold growth and unpleasant odors can also occur after leakage of pipes, presence of moisture in sub-floor crawl space or wet attics (Flannigan 1992).

Even with the advanced sampling and analysis equipment used today, it has not been possible to detect MVOCs from indoor air in concentrations equal to, or above the threshold limits for irritation (Wolkoff, 1997). Inadequate sampling techniques as well as inappropriate laboratory analyses could be the explanation. Although unequivocal evidence is lacking, field observations as well as results from experimental research, suggest that the actual indoor MVOCs may have some health implications (Larsen et al. 1998).

Actually we are dealing with different adverse health reactions to a large number of biologically active components. As long as the evidence for the connection between environmental findings and clinical symptoms is not sufficiently definite, working hypotheses include the possible different immunological and toxic effects.

Elucidation of the toxic and immunologic effects and risks connected with short- or long term- exposure to mold spores and their volatiles involves collaboration between researchers from many different disciplines. In addition to the health implications, there are many unsolved problems concerning intervention in water damaged buildings, e.g. the degree and extent of remediation and the economical consequences. Such collaborative tasks have been established in the elaboration of multidisciplinary mold programs in e.g. Finland and Denmark. These research programs comprise epidemiological and intervention studies aimed at the elucidation of the pathophysiological mechanisms and an identification of risk construction in buildings.

Moulds and health

The causal relation between presence of moulds and health effects should be elucidated and clarified. This will be done in a multicenter collaborative study

Human health effects from moulds

Establishment of better diagnostic in vivo and in vitro methods, especially for the non-atopic patients.

Characterization of moulds associated with damp buildings

Characterization of the funga will be performed by means of taxonomical and ecological features and conditions for amplification on materials.

Exposure to moulds in buildings - "From wall to nose"

Route of exposure to spores and metabolites on the human mucosa or skin after production, liberation, dispersal and deposition will be found.

Rapid detection methods for moulds in buildings

For technicians standard sampling methods for quick detection of moulds on building materials will be developed and validated.

Critical and safe building constructions in relation to dampness

To register current knowledge on mould growth in buildings a questionnaire will be send to all authorized Danish consulting architects and engineers.

Assessment of the condition of a mould infested building

For the consultants standard methods for estimation of the condition of a moldy building will be elaborated.

Resistance of building materials to mould attacks

The bio-availability of different organic building materials under different conditions of temperature and humidity will be studied.

Disinfection and remediation

Methods for physical, chemical and mechanical removal of moulds from building materials will be evaluated. Low risk materials and combination of materials with a low risk for mould attacks will be recommended.

IN ADDITION TO THE SCIENTIFIC PUBLICATIONS THE RESULTS OF THE PROJECTS WILL BE PUBLISHED AS REPORTS AND PRACTICAL RECOMMENDATIONS FOR REMEDIAL AND PREVENTIVE MEASURES.

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DURING REMOVAL OF BIOAEROSOL CONTAMINATED ROOF SUBSTRATE MATERIAL IN AN OCCUPIED FACILITY

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ABSTRACT

Visual observations and bulk sampling revealed that the four (4) yr. old plywood substrate of a failed roofing system would need to be removed and replaced. The microbial amplification which lead to the structural failure was likely caused by uncontrolled moisture migration in the humidified building. A plan was devised to establish and provide ongoing pressure containment and monitoring during removal and replacement of the entire roof from the top floor of a three story 40,000 sq.ft. facility. A replacement roofing system with adequate vapor and air barriers was designed and installed. Continuous multizone pressure monitoring and the use of real time laser particle counting during the renovation were planned to assure that containment of the construction zone was successful during the three (3) month removal and replacement process. Results confirmed that pressure containment to prevent intra-zonal transport of bioaerosols from the roof and fourth floor reconstruction area to the occupied floors below was successful. This approach allowed the project to be completed on schedule, within budget, and with little risk of occupant exposure to the large volume of contaminated materials that were removed.

KEY WORDS:

pressure containment, microbial aerosol control, decontamination, containment partitions, IAQ during renovation, occupied facility

INTRODUCTION:

During recent years, the authors have observed a growing concern of professionals within the construction industry for the health of occupants in facilities undergoing renovations. This has most recently been observed with the issuing of the guidance document in November 1995, entitled "IAQ Guidelines for Occupied Buildings Under Construction". This document was developed by SMACNA's (Sheet Metal and Air Conditioning National Contracting Association) Building Services Committee in cooperation with architects, engineers, and building owners and managers. The document is intended to present clear and concise manners and

methods to maintain acceptable indoor air quality in occupied buildings under construction.

During the fall of 1996 the authors encountered a challenging opportunity to apply these principles when they were asked to design repairs for a microbial contaminated roofing system in an occupied building. Initial visual observations revealed that the extent of the microbial amplification within the plywood substrate had rendered most of the four (4) year old material structurally unsound. Results of pressure and air pathway evaluations determined that the microbial amplification activity was located within the building envelope. In order to minimize possible occupant exposures, and to control principles of ACGIH Guidelines For the Assessment of Bioaerosols in the Indoor Environment (1989), it was determined that the vertically zoned HVAC equipment would need to be protected from contamination on the top floor via a combination of careful isolation of lower areas, and containment of the materials that would be disturbed in the attic and the top floor knee-wall areas.

MATERIALS AND METHODS:

Due to the condition of structural failure of the plywood from the microbial amplification activity, it was determined that the failed roofing system would need to be completely removed and replaced with a new design that would incorporate a continuous vapor and air barrier. Additionally, to effectively meet the previously noted SMACNA and ACGIH guidelines, (with sections of the roof missing), it was determined that a creative or novel pressure containment strategy would be needed.

HVAC/Building Shell Evaluation:

In order to determine the ability to effect a positive (+) pressure (isolation) in the occupied three lower level spaces during construction, a careful review and measurement of both the designed outside air and exhaust capacities for the facility was undertaken. In addition, intrazonal pressure mapping (with digital micromanometers) between the three lower floors and upper floor, and knee-walls were conducted. Results of the HVAC evaluation and pressure mapping evaluation revealed that the facility was zoned vertically with several floors served by the same air handlers, and that the building was designed to operate under a significant overall negative (-) pressure. Given this design, it was concluded that if disturbances of the contaminated substrate allowed materials to become airborne in the attic or on the fourth floor level, the emissions were destined for distribution to the remainder of the facility by the HVAC system.

Containment Design Approach:

The basic containment strategy that was determined to be most likely to succeed was to provide positive (+) pressurization of the lower three floors and to use the fourth floor as a partial containment zone during the roof renovation. Because of scheduling space needs, it was determined that only ½ of the fourth (4th)floor could be isolated at a time, necessitating a two (2) phase approach to the renovation. Through the use of ASTM Fan Pressurization testing of the facility, the amount of actual make up air that would be needed in order to positively (+) pressurize the lower three floors of the facility was determined. Analysis of the collected flow

vs. pressure data, allowed a permanent make-up air system to be designed and installed prior to the commencement of the roof and knee-wall renovation activities. Once the make-up air system was permanently installed and confirmed to work as designed, a phased pressure control strategy was planned. A temporary barrier (plywood and poly) was constructed at the top of the atrium stairwell to allow pressurization of the lower floors with respect to the upper floor and the outdoors. Additionally a temporary double wall vertical divider, (taped drywall and metal studs) was installed on the fourth floor. This double walled area allowed the top floor to be subdivided such that pressure control and containment could be maintained on ½ of the attic/knee-wall area for each of the two phases of construction. During construction, partial suction (-) was created on the attic and knee-wall areas with the combined use of: two (2) HEPA filtered negative air machines, manipulation of wall/ceiling leakage areas via openings, and manipulation of the supply and return volumes from the air handling units that serve the spaces.

RESULTS:

Air pressure data was recorded between the containment zone and isolation zone throughout the three month period via a dual channel strip chart recorder. Observation of the data generally revealed the desired continued positive (+) pressure relationships between the lower floors and the fourth during the three month life of the project, and the effects of wind on the facility. Additionally, Laser Particle Count data was logged via a portable computer for two size ranges, (greater than 0.5 microns diameter and greater than 5.0 microns diameter). An electronic time share device and custom designed valves allowed the Laser Particle Counter to collect data in two locations, (occupied zone and supply air duct). The particle count data revealed that periodic spikes of fine particle data were occasionally observed in the fourth floor occupied area that were simultaneous with increases in supply air particles, indicating a likely combustion source external to the building. Due to the need for instantaneous feedback on the effectiveness of the containment, real time pressure monitoring and laser particle counting was undertaken instead of airborne bioaerosol monitoring and laboratory processing.

DISCUSSION:

Results of three months of pressure monitoring reveal the general achievement of the desired pressure relationships; the occupied isolated space being kept most positive, and the contained construction areas being kept most negative, with a buffer zone in between. Results of the laser particle counting confirm continuous delivery of clean air to the isolation areas, and also identified disruptions in the containment zone caused by local construction and furniture moving.

CONCLUSION:

Pressure and laser particle count monitoring revealed continuous negative pressure containment (-) and occupied floor positive pressure (+) isolation was successful. This program appeared to prevent intra-zonal transport of bioaerosol contaminated air from the roof recon-

struction area to the occupied floors below. This real time monitoring approach allowed the project to be completed on schedule with little occupant risk of exposure from the large quantity of bioaerosol contaminated materials that were disturbed and removed from the occupied facility.

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CHARACTERIZATION, PREVENTION, AND CONTROL OF MILDEW IN RESIDENTIAL ENVIRONMENTS: AN APPLIED RESEARCH STUDY

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ABSTRACT

A 10-week, 10-home cleaning and mold and mildew prevention study was conducted in humid North Carolina to: 1) characterize "mold and mildew" on hard surfaces associated with moisture, and 2) assess efficacy and frequency of application of a disinfectant product in the treatment group (5 homes) compared to the control group (5 homes). After professional cleaning and standardization of cleaning products and vacuum cleaners, a total of 24 hard surface sites were selected, with each sampled twice per week in all 10 homes. Sites in intervention homes were treated with a spray disinfectant after each sampling and on one additional day (3 times per week total). Samples (4,800) were processed for culturable fungi (molds and yeasts). Sites included window frames, sink/tub faucets, toilet-floor and tub-floor connections, under sink plumbing and cabinets, dishwasher vent, floor under washing machine, laundry room walls, shower curtain liners, and trash cans. More than 30 different fungi were recovered, many of which are considered potentially allergenic, toxigenic, and opportunistic. They included species of *Cladosporium, Penicillium, Aspergillus, Fusarium, Aureobasidium, Paecilomyces, Alternaria, Trichoderma, Phialophora, Ulocladium, Stachybotrys/ Memnionella, Wallemia* and *Acremonium*. Mold and mildew contamination was significantly reduced in treatment homes where hard surfaces were routinely disinfected, compared to controls. The importance of both cleaning and disinfection in the maintenance of a healthy home environment was demonstrated.

KEY WORDS:

mold & mildew, mildew reservoirs, mildew characterization and control, mold & mildew disinfection, mildew organisms identified

INTRODUCTION

Mildew in home environments is considered an aggravating factor for respiratory allergies, as well as a cause of damage to building materials (Flannigan and Miller, 1994). Each indoor home environment supports a microbial ecology that can influence the quality of the indoor air (Cole et al. 1994). The indoor environment is an ecosystem comprised of an interrelated

series of microenvironments, each of which can serve as a reservoir of microbial contamination. If not controlled, these can become a source of biopollution (Cole et al. 1996). Such reservoirs are usually associated with moisture, resulting in the growth, amplification, and airborne dissemination of environmental fungi (Pasanen, 1992) that may be allergenic, toxigenic, and/or opportunistic. Objectives of the study were to characterize "mold & mildew" on hard surface sites throughout the home environment, and to assess the effectiveness of the routine use of a spray disinfectant in controlling fungal growth on those sites.

METHODS AND MATERIALS

Following preliminary assessment of hard surface sites in home environments, 24 areas associated with moisture were selected for characterization and control. The study was conducted in 10 condominium homes in Raleigh, North Carolina from August through October 1997. Participant homes were microbially characterized to rule out problem indoor environments, professionally cleaned, and randomly assigned to control and treatment intervention groups. All were given the same cleaning products to use at normal frequency, to include a high-efficiency vacuum cleaner.

All 24 hard surface sites were sampled twice per week in all homes. Sites in intervention homes were treated with a quaternary ammonium alcohol-based spray disinfectant (Lysol® Brand II) after each sampling and on one additional day for a total of 3 times per week. A total of 4,800 samples were collected and processed for culturable molds and yeasts. Sites included window frames, sink/tub faucets, toilet-floor and tub-floor connections, under sink plumbing and cabinets, dishwasher vent, floor under washing machine, laundry room walls, shower curtain liners, and trash cans.

Samples were collected using sterile moist swabs. Each swab was used to cleanse the surface of an area previously defined by discrete measurements or by the use of specific-sized templates. In the laboratory, swabs were eluded and plated on three isolation/identification media (Gibson Laboratories, Lexington, KY): 2% Malt Extract Agar (BBL) with 0.05% chloramphenicol, Dichloran-Glycerol Agar (DG18), and Potato Dextrose Agar with 0.05% chloramphenicol. All media were incubated at room temperature (23-25°C) under alternating conditions of light and dark for 7-10 days prior to final quantitation and identification. Data were organized and analyzed as total colony-forming units (CFU) for each site in all control homes versus CFU for each site in all treatment homes, and also on the basis of mean CFU/cm^2 for each site for all control homes versus the same sites for all treatment homes. An assessment of rank order predominance was also performed for each of the 24 sites in each group.

Statistical analysis was performed using a two step approach: 1) descriptive analysis that investigated the basic structure, distribution, and overall dispersion of the data based on the second, third, and fourth moment around the mean, 2) a repeated measure design using the Generalized Linear Model (GLM) procedure in SAS© which provided both MANOVA and univariate ANOVA results.

RESULTS

Results from the 24 surface sites in 10 study homes showed "mold & mildew" to consist of more than 30 different fungi (molds and yeasts), many of which are considered potentially allergenic, toxigenic, and opportunistic. These included species of *Cladosporium*, *Penicillium*, *Aspergillus* (including *A. versicolor*, *A. niger*, *A. ochraceus*, *A. flavus*, *A. fumigatus*, *A. glaucus*, *A. terreus*), *Fusarium*, *Aureobasidium*, *Paecilomyces*, *Alternaria*, *Trichoderma*, *Phialophora*, *Ulocladium*, *Stachybotrys/Memnoniella*, *Wallemia*, *Acremonium*, and *Rhodotorula* and a variety of other yeasts (described by their pigmentation). Fungi isolated from all sites in all control homes, with their rank order of predominance is shown in Table I.

TABLE I: Fungi in Rank Order Isolated From Hard Surfaces Associated with Moisture in Non-problem Home Environments.

Cladosporium sp.
Cream yeast
Rhodotorula glutinis
Aureobasidium pullulans
Penicillium sp.
Fusarium sp.
Aspergillus versicolor
Pink yeast
Ulocladium sp.
Black yeast
Aspergillus sp.
Alternaria sp
Phialophora sp.
Aspergillus flavus
Paecilomyces sp.
Aspergillus niger
Aspergillus glaucus
Dematiaceae
Stachybotrys/Memnoniella sp.
Aspergillus ochraceus
Aspergillus fumigatus
Trichoderma sp.
Wallemia sebi
Acremonium sp.
Aspergillus terreus
Chaetomium sp.
Neurospora sp.
Rhizopus sp.
Aspergillus clavatus
Mucor sp.
Other

The effectiveness of the spray disinfectant was demonstrated as mold and mildew contamination was significantly reduced in treatment homes where hard surfaces were routinely disinfected compared to control homes without disinfection. Least square means analysis demonstrated that the treatment group had a significantly lower CFU/cm^2 count than the control group over time. The most significant ($p<0.001$) and dramatic treatment effects for reduction of fungi CFU/cm^2 were found at the following sites: all window frames, half-bathroom sink edge, half-bathroom sink faucet, the floor under the washing machine, the living room patio door frame, the master bathroom sink faucet, the kitchen sink edge, around the kitchen sink faucets, and the bottom of the kitchen trash can. Residual predominant fungi in rank order from all treatment homes were: *Penicillium* sp., *Aspergillus versicolor*, *Cladosporium* sp., cream yeast, *Aspergillus* sp., *Fusarium* sp., *Rhodotorula*, and representatives of most of the other fungi found in the control homes.

DISCUSSION

The study results have assisted in the characterization of the microbial ecology of indoor residential environments and defined "mold and mildew" from hard surface sites associated with moisture as a variety of potential allergenic, toxigenic, and opportunistic organisms. While disinfectant treatment effectively resulted in reservoir reduction, residual organisms (perhaps with reduced sensitivity) were primarily *Penicillium* and *Aspergillus*, emphasizing the importance of both cleaning and disinfection in the maintenance of a healthy home environment.

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ACGIH TLV STATEMENT ON BIOAEROSOLS; AMERICAN COUNCIL OF GOVERNMENT INDUSTRIAL HYGIENISTS PRESENTED FOR THE BIOAEROSOLS COMMITTEE BY

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Biologically Derived Airborne Contaminants. TLVs exist for certain substances of biological origin, including cellulose; some wood, cotton, and grain dusts; nicotine; pyrethrum; starch; subtilisins (proteolytic enzymes); sucrose; and vegetable oil mist. However, for the reasons identified below, there are no TLVs against which to compare environmental air concentrations of most materials of biological origin. The ACGIH Bioaerosols Committee has developed and separately published guidelines to assess, control, remediate, and prevent biologically derived contamination in indoor environments.

For the purposes of the guidelines, indoor biological contamination is defined as the presence of:

a) biologically derived aerosols of a kind and concentration likely to causes disease or predispose people to disease;

b) inappropriate concentrations of outdoor bioaerosols, especially in buildings designed to prevent their entry; or

c) indoor microbial growth and remnants of biological growth that may become aerosolized and to which people may be exposed.

The guidelines define an approach to assessing and controlling bioaerosol exposures. This approach relies on visually inspecting buildings, assessing occupant symptoms, evaluating buildings performance, monitoring potential environmental sources, and applying professional judgement.

Biologically derived airborne contaminants include bioaerosols (airborne particles composed of or derived from living organisms) and volatile organic compounds that organisms release. Bioaerosols include microorganisms (i.e., culturable, non-culturable, and dead microorganisms) and fragments, toxins, and particulate waste products from all varieties of living things. Biologically derived contaminants are ubiquitous in nature and may be modified by human activity. All persons are repeatedly exposed, day after day, to a wide variety of such materials.

The guidelines provide background information on the major groups of bioaerosols including their sources and health effects. The guidelines also describe methods to collect, analyze, and interpret bioaerosol samples from potential environment sources. Occasionally, environmental monitoring detects a single or predominating biological contaminant. More commonly, monitoring reveals a mixture of many biologically derived materials, reflecting the diverse and interactive nature of indoor microenvironments.

Environmental sampling for bioaerosols should be conducted only following careful formulation of testable hypotheses about potential bioaerosol sources and mechanisms by which workers may be exposed to bioaerosols from these sources. Even when investigators work from testable hypotheses and well-formulated sampling plans, results from environmental bioaerosol monitoring may be inconclusive and possibly misleading.

For the reasons identified below, there are no TLVs for interpreting environmental measurements of:

a) total culturable or countable bioaerosols (e.g., total bacteria or fungi);

b) specific culturable or countable bioaerosols (e.g., *Aspergillus fumigatus*);

c) infectious agents (e.g., Legionella pneumophila, Mycobacterium tuberculosis); or

d) assayable biological contaminants (e.g., endotoxin, mycotoxin, antigens, or microbial volatile organic compounds).

A. Total culturable or countable bioaerosols. Culturable bioaerosols are those bacteria and fungi that can be grown in laboratory culture. Such results are reported as the number of colony-forming units. Countable bioaerosols are those pollen grains, fungal spores, bacterial cells, and other material that can be identified and counted by microscope. A general TLV for culturable or countable bioaerosol concentrations is not scientifically supportable because of the following:

   1) Culturable microorganisms and countable biological particles do not comprise a single entity, i.e., bioaerosols in occupational settings are generally complex mixtures of many different microbial, animal, and plant particles.

   2) Human responses to bioaerosols range from innocuous effects to serious, even fatal, diseases, depending on the specific material involved and workers' susceptibility to it. Therefore, an appropriate exposure limit for one bioaerosol may be entirely inappropriate for another.

   3) It is not possible to collect and evaluate all bioaerosol components using a single sampling method. Many reliable methods are available to collect and analyze bioaerosol materials. However, different methods of sample collection and analysis may result in different estimates of the concentrations of culturable and countable bioaerosols.

   4) At present, information relating culturable or countable bioaerosol concentrations to health effects is generally insufficient to describe exposure/response relationships.

B. Specific culturable or countable bioaerosols other than infectious agents. Specific TLVs for individual culturable or countable bioaerosols have not been established to prevent hypersensitivity, irritant, or toxic responses. At present, information relating culturable or countable bioaerosol concentrations to health effects consists largely of case reports and qualitative exposure assessments. The data available are generally insufficient to describe exposure/response relationships. Reasons for the absence of good epidemiologic data on such relationships include the following:

   1. Most data on concentrations of specific bioaerosols are derived from indicator measurements rather than from measurements of actual effector agents. For example, investigators use the air concentrations of culturable fungi or represent exposure to airborne fun-

gal antigens. In addition, most measurements are from either area or source samples. These monitoring approaches are less likely to reflect human exposure accurately than would personal sampling for actual effector agents.

2. Bioaerosol components and concentrations vary widely within and among different occupational and environmental settings. Unfortunately, replicate sampling is uncommon in bioaerosol assessments. Further, the most commonly used air sampling devices for indoor monitoring are designed to collect "grab" samples over relatively sort time intervals. Measurements from single, short-term grab samples may be orders of magnitude higher or lower than long-term average concentrations and are unlikely to represent workplace exposures accurately. Some organisms and sources release aerosols as "concentrations burst," which may only rarely be detected by limited grab sampling. Nevertheless, such episodic bioaerosol releases may produce significant health effects.

C. Infectious agents. Human dose/response data are available for only a few infectious bioaerosols. At present, air sampling protocols for infectious agents are limited and suitable primarily for research endeavors. In most routine exposure settings, public health measures, such as immunization, active case findings, and medical treatment, remain the primary defense against infectious bioaerosols. Facilities associated with increased risks for transmission of airborne infectious disease (e.g., microbiology laboratories, animal handling facilities, and health care settings) should employ engineering controls to minimize air concentrations of infectious agents. Further, such facilities should consider the need for administrative controls and personal protective equipment to prevent the exposure of workers to these bioaerosols.

D. Assayable biological contaminants. Assayable, biologically derived contaminants (e.g., endotoxin, mycotoxins, antigens, and volatile organic compounds) are microbial, animal, or plant substances that can be detected using chemical, immunological, or biological assays. Evidence does not yet support TLVs for any of these substances. However, assay methods for certain common airborne antigens and endotoxins are steadily improving, and field validation of these assays is also progressing. Dose/response relationships for some assayable bioaerosols have been observed in experimental studies and occasionally in epidemiologic surveys. Therefore, TLVs for some of these substances may be appropriate in the future. Also, innovative molecular techniques are becoming available for specific bioaerosols currently detectable only by culture or counting.

ACGIH actively solicits information, comments, and data that will help the Bioaerosols Committee evaluate the potential for health effects associated with bioaerosol exposures in occupational and related environments. Such information should be sent to ACGIH Technical Affairs office.

REFERENCE

- ACGIH (American Conference of Governmental Industrial Hygienists). 1989. Guidelines for the Assessment of Bioaerosols in the Indoor Environment. Cincinnati, OH

INDUSTRIAL HYGIENE & CLEARANCE CONSIDERATIONS FOR A MICROBIAL REMEDIATION PROJECT

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ABSTRACT:

A general consensus on final clearance levels for microbial remediation projects does not exist. No numeric standards for microbial sampling exist. In-depth microbial surveys should determine the full extent of contamination and which areas do not need abatement or only need local abatement. To pass final clearance, 3 parameters should be used: visual inspection, and results of air and swab sampling. In the authors' projects, spore trap samples were collected as a screening test prior to final clearance samples (air and swab). If the spore traps show that work areas were not sufficiently cleaned, the areas were re-cleaned. The authors have broken down projects into smaller work zones to separate more heavily contaminated zones from less contaminated zones. On-going clearance testing as areas were finished sped up the abatement. Procedures such as localized remediation within fully contained areas limited microbial release inside the work area and helped achieve final clearance faster.

KEY WORDS:

Final Clearance, Indicator Fungi, Industrial Hygiene, Localized Remediation, Microbial Abatement, Remediation, Spore Traps

How to set a final clearance level(s) for a microbial remediation project is not yet a clear-cut process, nor is there a general consensus in the industrial hygiene or IAQ community. This paper is based on the authors' experiences over the past 6 years with 35 microbial abatements in various buildings: office spaces, libraries, universities, high schools, homes, and a detention center. Some of the abatement projects were undertaken in only specified part(s) of the building (i.e., offices and schools), but most projects included the entire building. Three particular cases involved new buildings which had not yet been occupied. The authors' approach has changed during this period, due to advances in understanding abatement protocols from the IAQ community and from our own "on-the-job" experiences. In addition to final clearance considerations, this paper will also discuss "learned on-the-job" procedures to limit the release of microbials inside, as well as outside, the work project area.

While there are some microbial abatement guidelines (New York State Department of Health, 1995; Light et al. 1989; Johanning et al. 1993), as well as asbestos abatement protocols to follow, there are many unique aspects to a microbial abatement project. We also believe that the final clearance strategy for any specific building should be based on how the initial microbial

cleaned. If a work zone consisting of 16 small offices has, for example, only one room that does not pass, that room is re-cleaned rather than the entire work zone. These areas are then re-sampled by the environmental consultant.

PLANNING AN ABATEMENT PROJECT

When planning an abatement project, the authors' strategy, when applicable, is to break down the work project into smaller work zones. This has several advantages. It can separate more heavily contaminated work zone(s) from less contaminated zone(s). By specifying that these areas must be isolated from each other, it lessens the risk of spreading the fungi from major contaminated areas during the actual abatement to other less contaminated areas. In addition, to pass a final clearance in an entire building is not easy. In our experience, few of the abatements passed final clearances in all work zones after the first round of sampling. Typically, however, the less contaminated work zones do pass the first final clearance.

A building often needs major re-construction after an abatement, and the quicker a floor or a section of a floor can undergo "re-building", the faster it comes back on-line. In the 3 libraries, the basement was clearly more contaminated than the 1st floor. By having the 1st floor completely sealed off from the basement and by using separate decontamination units for each floor, each library was able to start renovation on the 1st floor even while the basement was undergoing an additional cleaning and final clearance. If the entire building had been considered one work zone, up to a month would have been lost for the start of the re-construction process.

On one large building abatement, 12 individual work zones were established, and the abatement lasted for 10 months. Typically, not all sections (work zones) of the building can be abated at the same time. The authors have successfully used a "screening" final clearance as each work zone was completed. This screening was performed only using swabs (no air samples were collected). The swabs from the work zone(s) were collected as a screening test before final clearance sampling was performed.

Another similar approach that the authors have recently used on projects is spore trap air sampling. Spore trap air sampling will reveal both viable and non-viable microbials, and also has a quicker turn-around time when compared to culturable swabs. The samples are analyzed by microscopy methods, and concentration levels are reported in fungal structures/m^3. Identification is presumptive for any individual fungi, due to the limitations of microscopy methods, but it gives a better picture of total fungal contamination than any culturable analysis method.

The spore trap samples are used as a screening test prior to any culturable sampling (air or swab). If the spore trap samples show that the work area(s) are still not sufficiently cleaned, these work area(s) will be re-cleaned and re-sampled. Airborne fungal types found in the work area samples from inside the containment area should be qualitatively similar to that of outdoor air. Also, the screening level concentrations should not to exceed the presence above background of outdoor air concentration levels for any marker (indicator) fungi. Outdoor air spore trap samples should be taken in addition to any indoor air spore trap sampling.

During the six projects where spore trap sampling was performed, culturable air and swab samples were also collected at the same time. In most cases, the agreement with the spore trap results and the culturable results was good. However, not all "positive" spore trap data showed up in the culturable samples (as expected since some spores are not viable). Also, not all positive culturable sample date yielded similar results for spore trap samples (e.g., *Stachybotrys chartarum* was detected in culturable samples, but not in spore traps). The authors believe spore trap sampling is a useful (and quick) tool, but should not be used as a sole source for determining final clearance.

The authors used the swab and/or spore trap lab results as a "screening" test to decide whether to proceed with final clearance testing (which consists of both culturable air and swab samples, with more swabs collected than in the "screening" test). If the work zone does not pass the "screening" tests, then it certainly would not pass the building-wide "final" final clearance sampling. Any final clearance sampling would be more strenuous than the initial screening test. If a work zone passes this screening test, then the area is sealed off. If it does not pass the screening test, then it is re-cleaned.

This "screening" approach can save time and cost for the client. For large projects, having a "screening" final clearance for each work zone as abatement proceeds in other work zones accomplishes several goals. (Anon (1993) Usually some pattern can be seen in the abatement procedure, such as inadequate cleaning of floors and/or crawl spaces and/or piping; and the environmental consultant can re-direct attention to these deficiencies. (Institute of Medicine (1993) If the work zone(s) does not pass the "screening" final clearance, the logistics of re-mobilizing the work crew does not present a problem as they are still on site. (Johanning et al. 1993) It enables both the abatement work crew and the environmental consultant to get a sense of what it actually takes to achieve final clearance. We find the work improves after the work crew actually sees what "clean" means in a microbial abatement.

These various factors all save time; a definite consideration when the turn-around time for analysis is, at best, 2 weeks. If "screening" is not used, the down time between rounds of final clearances loom larger and larger. The owner/tenant wants the building back in service as soon as possible and "screening" helps to achieve that goal.

The authors do not recommend that a passed work zone on a large project remain under negative pressure; it has been our experience that initial gross removal activities in near-by work zones will spread mold into the cleaned zone via the negative pressure in the cleaned work zone. Instead, we recommend that the work zone(s) that pass the screening test be kept sealed.

After all abatement activities are complete and the screening tests are passed, the entire building should undergo a "final" final clearance. The authors believe that most work zones would fail without this screening process. Our experience in using "screening" tests has been, instead, that almost all work zones pass building-wide final clearance. If, for example, 9 out of 12 work zones pass finals, but 3 do not, only a part of the building must undergo re-cleaning instead of the entire building.

Careful planning is required when using multiple work areas. Normally the differential pressure of the work area is maintained at a minimum of 0.03 inches of water pressure below that of adjacent clean areas (or negative to the clean areas). Manometers are placed in the decontamination unit clean room and adjacent areas to verify compliance. When adjacent work areas are

remediated simultaneously, at some point one work area will become cleaner than the other. If the pressure differential of the cleaner area is more negative than the adjacent work area, microbial contamination can be drawn into the clean area. Wherever possible the authors require a buffer area between work areas to avoid this scenario.

At the 3 library projects, time constraints required the adjacent first floor and basements to be abated in all 3 libraries simultaneously. The basement was clearly the more contaminated area and required significantly more demolition activities. The differential pressure between the ambient environment and the basement was maintained at -0.03 inches of water; and the differential between the ambient environment and the first floors was maintained at -0.02 inches of water. This minimized the possibility that if a pathway existed, air would flow to the first floor from the basement. As a further precaution, final cleaning could not commence in either area until all demolition activities were complete and the work areas were substantially dust free.

We have also used localized remediation within a fully contained area for removing materials which are considered to be significantly microbially contaminated and for which the release of microbials can not easily be controlled; for example, contaminated cementitious pipe fitting insulation which must be wire brushed to be completely removed, or a small area of water-damaged ceiling plaster. Sealed tents lined with 2 layers of plastic sheeting (6-mil thickness at a minimum) and fully framed with wood or metal studs are used. Negative air within the tent is utilized. After removal, a thorough cleaning of the tent must be done. Tents may not be dismantled until a visual inspection is performed by the environmental consultant and a minimum of ten full air changes have occurred inside the tent (after removal of contaminated materials).

The authors' approach towards microbial abatement has changed over the past 4 years, and the authors do not believe the final word has been spoken for final clearance and remediation methods.

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MICROBES AND MOISTURE CONTENT OF MATERIALS FROM DAMAGED BUILDING

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ABSTRACT

The aim of the study was to characterize the microbial flora of the envelope of a school building and the correlation of the microbial concentrations and the moisture content of materials was estimated. Material samples (n=95) were taken from different structures before the dismantling of the school. In all, 40 sample pairs were taken from which both concentrations of microbes and moisture contents (% by weight) were determined. The range of the total concentration of fungi was <45 - 8 600 000 cfu/g for all the analyzed material samples. The growth of microbes in a building is mostly regulated by the moisture of the materials and often the elevated concentrations of mesophilic fungi (>10 000 cfu/g) were associated with elevated moisture content of the material. However, the microbial concentrations did not fully correlate with the moisture contents of the material.

KEY WORDS:

building materials, microbes, moisture

INTRODUCTION

The aim of the study was to characterize the microbial flora of the building using material samples. The moisture content (RH) of materials was also determined to study the correlation between microbial concentration and moisture contents. The study site was a high school building. The building was built in 1968, originally for temporary use. The building had moisture problems and it was demolished in autumn 1995. Material samples were taken from different layers of the base floor, inside and external walls and the ceiling structures before and during the dismantling.

MATERIAL AND METHODS

ANALYSIS OF MICROBES

In all, 95 samples were taken in parallel with the dismantling so that different layers of each material could be sampled. In the laboratory the samples were crushed, soluted into a sterile dilution water and the dilution series were made. The samples were plated both on 2% malt extract agar (M2) and dichloran-18-glycerol agar (DG18) for viable fungi and on tryptone-glucose-yeast agar for bacteria. The samples were incubated at 25°C for 7 days for fungi and at 20°C for 5 and 14 days for bacteria and actinomycetes. The results were calculated in relation to the material's dry weight.

ANALYSIS OF MOISTURE CONTENTS

Moisture contents were determined for forty corresponding wooden and insulation wool materials from the base floor and wall structures. Moisture contents were determined in percentage by weight using oven-drying method and then converted to relative humidity (RH) using the sorption diagrams.

RESULTS

The concentrations of mesophilic fungi for all the analyzed material samples varied from under the detection limit (45 cfu/g) to 8 600 000 cfu/g. When the concentrations were over 10 000 cfu/g, following microbes were detected: *Acremonium, Aspergillus, Aureobasidium, Hyalodendron, Mucor, Oidiodendron/Geomyces, Olpitrichum, Penicillium, Phialophora, Sphaeropsidales*-group, non-sporing isolates, *Wallemia* and yeasts.

In the wooden external and inside walls were no visible moisture damages, and the relative humidity of the materials was at maximum 79 %. The number of the mesophilic fungi were <45 - 5 600 cfu/g, consisting mainly of *Penicillium*, *Aspergillus*, yeasts, non-sporing isolates, *Oidiodendron, Aureobasidium* and *Sphaeropsidales*-group. In the base floor structures, massive visible damage could be seen. The highest moisture content (RH) for both the wooden and insulation wool structures was 93 %. The range of the fungal concentrations was from < 45 to 590 000 cfu/g for the wooden structures and from < 45 to 5 900 000 cfu/g for the insulation wools. The most common fungi in materials were *Penicillium*, *Aspergillus*, *Cladosporium*, non-sporing isolates, *Acremonium, Hyalodendron, Oidiodendron/Geomyces* and *Sphaeropsidales*-group. *Wallemia, Phialophora, Polyscytalum, Olpitrichum, Mucor* and *Trichoderma* were also found.

In many cases the elevated concentrations of mesophilic fungi (>10 000 cfu/g) were associated with elevated moisture content of the material (above the normal moisture content of the structural element in the operating conditions). As a whole the linear correlation was not found between the microbial concentrations and the moisture contents of the materials.

DISCUSSION

The growth of microbes in a building is mostly regulated by the moisture of the materials. In the samples taken from the moisture damaged places the concentrations of mesophilic fungi were high, > 10 000 cfu/g. Yeasts, *Phialophora*, *Wallemia* and *Mucor*, which are regarded as moisture indicators (Samson et. al 1994, ISIAQ 1996), were detected from the samples. *Oidiodendron/Geomyces* that appeared in the samples, has earlier been detected in air samples from moisture damaged buildings (Hyvärinen et al. 1993, Meklin et al. 1996). However, the microbial findings did not follow the results of the moisture measurements. This is due to the complex nature of moisture behavior of a building and its different structures. Moisture content of structures depend on moisture load, which changes with diurnal as well as with seasonal rhythm. Also structures affect the moisture physical behavior. For example, wall structures, were prone to cyclic wetting and drying with the weather conditions. The samples were taken just after summer when the walls were dry while the microbial concentrations in separate samples indicated either mould damage or the presence of normal background flora. The floor structures kept their moisture continuously due to progressive moisture load from the ground.

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USE OF MVOC MEASUREMENTS AND ODOR PERCEPTION AS INDICATOR OF MOULD IN INDOOR AREAS.

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ABSTRACT

Moulds are amongst the most important indoor allergens. Mould formation in buildings and residential housing is often caused or facilitated by construction defects and lack of building use. Moulds produce a range of volatile organic compounds, the Microbial Volatile Organic Compounds (MVOCs). Some of these compounds, such as 1-octene-3-ol, 2-methyl-1-propanol, dimethylsulphide, dimethyldisulphide, dimethylsulphoxide, 2-heptanone and geosmin, produce a musty, foul or earthy odor which can be detected long before any visible signs of mould growth are evident.

In small test samples (between 1and 5 liters) thermodesorption combined with mass spectrometry allows an analytical detection of these compounds in indoor air at concentrations substantially lower than their olfactory threshold. For these analyses, only small test samples (1 to 5 liters) are required.

Result of this study: 131 residences were analyzed. In all rooms (n=96) with olfactory signs of mould formation, more than 50 ng/m^3 of MVOC's could be detected.

MVOC is sensitive, specific, valid and thus helps to prevent exposure to fungal allergens.

INTRODUCTION

Damp building materials represent the main cause of microbial growth (moulds, bacteria). Residents of damp rooms, contaminated with moulds have a variety of symptoms indicating allergic reactions of skin, eyes and the bronchial system (Senkpiel, Ohgke, 1996). Apart from allergic reactions, infections can be caused in patients with immune defects, especially by thermotolerant *Aspergillus* species.

In many cases, the growth of mould is not visible in suspected rooms. Air sampling for fungal spores may not be successful in tracing the allergenic source. Metabolic products of moulds, however, may be smelled by characteristic odor (Table I). The odor is composed of microbial volatile organic compounds (MVOC's).

The aim of this study was to determine the extent to which the perception of mould odors by untrained individuals correlated with the measured concentrations of both MVOCs and fungal spores in air.

TABLE I: MVOCs and their olfactory threshold concentrations (1).

MVOCs	Olfactory threshold µg/m³	Odor
methylisobutylether		
methylisopentylether		
2-methylfuran	90,000	
3-methylfuran*		
2/3-methyl-1-butanol*	45/30	sour and sharp
2-methyl-1-propanol	3	musty, fungus-like
1-decanol*	0.6	
1-octen-3-ol*		fungus-like
3-octanol*		
3-octanone*	30,000	mild and fruity
2-butanone	740	sweet and sharp
2-heptanone*	94	fungus-like and musty
dimethylsulphide*	2	rotten and decayed
dimethyldisulphide*	0.1	uncomfortable
dimethyltrisulphide*	6	foul
dimethylsulphoxide*		musty
β-farnesene		
geosmin	7	earthy

* these compounds were included amongst the total MVOC values as listed in Table IV

MATERIALS AND METHODS

Rooms both with (n=117) and without (n=14) suspected mould growth were investigated in this study. Residences with mould were subcategorised according to Deweg et al. (Deweg et al. 1995), taking into account whether visible cases of mould-induced damage were light or severe, or whether superficial measures had been undertaken to treat earlier cases of mould growth. The classification criteria for both the extent of damage and the perception of mould-derived odors are given in Table II. These criteria were applied by the different people who took the samples. The odor impression was noted on a three-stage psychometric scale. All the rooms were perceived by the inhabitants to have musty, fungus-like odor.

TABLE II: Mould exposure in indoor areas - classification criteria for the extent of damage and perception of odor (according to [2, 5]).

A = Dry residences
B = Residences where mould damage was only superficially sanitized (removal of visible mould growth)
C = Cases of mould growth suspected due to the presence of odors or construction defects (leakages with water entry or breaks in the heat insulating layers)
D = Visible, light cases of mould growth
E = Visible, extensive cases of mould growth

1 = No mould odor
2 = Light or moderate mould odor
3 = Very strong mould odor

For mycological analysis, 150 litre air samples were taken over 3 minutes with an slit impactor (LORECO, FH2) using Sabouraud (4%) glucose agar (SA-agar) or malt-extract agar (Senkpiel, Ohgke, 1994).

For the MVOC samples, between 1 and 5 litre samples of air were collected through a 60/80 mesh tenax TA before thermodesorption (Gerstel, TDS 3) coupled to analytical gas-chromatography with mass-spectrometry (GC-MS system, Hewlett Packard, 6890) (Keller, Sönnichsen, 1996; Keller et al. 1997; Sönnichsen, Keller, 1997).

The rooms under investigation were not mechanically ventilated. The residents were instructed not to open the windows for at least 24 hours before sampling.

RESULTS AND DISCUSSION

One hundred and seventeen (117) residences with varying degrees of mould exposure were investigated together with 14 control residences. Of the 131 residences which were investigated, 9 showed an extensive, visible degree of mould exposure on one or several walls. In eight of these residences a strong mould odor was apparent (Table III). Seven of the residences with superficially removed mould growth (group B in Table III) had extensive amounts of fungus on the walls until a few weeks before the study was conducted. These were removed mechanically before the wall surfaces were treated with fungicide containing paint.

TABLE III: Mould growth and odor in the indoor areas studied (n=131).

	Mould odor			
	none	**light**	**strong**	**total**
Extent of damage	**Number of indoor areas investigated**			
A = Dry residence	14	0	0	14
B = Superficially sanitized	7	12	0	19
C = Presumed but not visible	11	52	15	78
D = Visible but not extensive	3	8	0	11
E = Visible and extensive	0	1	8	9
Total	35	73	23	131

Comparisons between the olfactory detection of mould and both the measured mould spore and mould MVOC concentrations as related to the extent of damage. The extent of damage was classified semi-quantitatively according to Table IV. A strong mould type odor was only detected when extensive damage had occurred and mould spore concentrations were substantially higher than reference values (outdoor air).

TABLE IV: Mould exposure, the presence of MVOCs and total spore concentrations expressed as ratios of indoor to outdoor values (I/O*).

	Mould spore concentrations (Indoor air/outdoor air - I/O*) and total MVOC's					
Mould odor	**None**		**Light**		**Strong**	
	Spores	**MVOC's (ng/m³)**	**Spores**	**MVOC's (ng/m³)**	**Spores**	**MVOC's (ng/m³)**
Extent of damage						
A = Dry residence	I/O = 0.7	< 10		n.d.		n.d.
B = superficially sanitized	0.8	< 10 - 30	1.0	65 - 210		n.d.
C = presumed but not visible	1.0	15 - 25	1.0	50 - 920	1.0	160 - 3800
D = visible but not extensive	1.0	10 - 35	1.0	145 - 1720		n.d.
E = visible and extensive		n.d.	2.5	920	4.0	250 - 12300

Outdoor air = MVOC: <10 ng/m³ (n=36)
n.d. = not done
I/O = ratio of the number of spores detected indoor vs. Number of spores detected outdoor
The count of outdoor spores had a range between 125/m³ and 283/m³

Spores: The collected fungal spores were identified on a genus niveau. They belonged mainly to *penicillium spp.* and *aspergillus spp. Stachybotrys* was not found.

Analysis of the summed concentrations of the MVOC compounds selected for study (Table III) revealed that values of up to 35 ng/m³ could be measured before any mould odor was apparent (n=35 residences). With perception of only a light odor, total MVOC concentrations of between 50 and 1720 ng/m³ were reported (n= 73 residences). Only at high MVOC concentrations between 160 and 12300 ng/m³ a strong mould odor could be detected.

For residences in which mould presence was presumed although visible signs were not apparent (representing the largest group in this study), a summed MVOC concentration of 50 ng/m³ provides reason to suspect the presence of non-visible mould growth, as long as the summed MVOC concentrations measured in outdoor air are $\leq$ 10 ng/m³ as could be shown in this study. A comparison of these results with similar studies is difficult as no standardized and validated methods for measurement or classification are yet available.

CONCLUSIONS

As long as MVOC concentrations measured outdoors do not exceed 10 ng/m³, indoor MVOC concentrations above 50 ng/m³ can already indicate the presence of non-visible mould growth in naturally ventilated indoor areas and explain the appearance of a light mould odor.

This study shows that it is appropriate to measure MVOC when mould growth is suspected but not yet visible or to be smelled. For evaluating the health effects of indoor mould growth a mycological investigation is required which should also consider the simultaneous presence of raised spore concentrations in outdoor air. For a complete evaluation of health effects, information concerning the presence of mesophilic (allergenic risk), thermotolerant (allergenic and infectious risk) and thermophilic actinomycete (risk of an exogenous-allergic alveolitis) spores and a medical investigation is also required.

SUMMARY

Moulds are amongst the most important indoor allergens. Mould formation in buildings and residential housing is often caused or facilitated by construction defects and lack of building use. Moulds produce a range of volatile organic compounds, the so-called Microbial Volatile Organic Compounds (MVOCs). Some of these compounds, such as 1-octene-3-ol, 2-methyl-1-propanol, dimethylsulphide, dimethyldisulphide, dimethylsulphoxide, 2-heptanone and geosmin, produce a musty, foul or earthy odor which can be detected long before any visible signs of mould growth are evident.

In small test samples (between 1and 5 liters), thermodesorption combined with mass spectrometry allows an analytical detection of these compounds in indoor air at concentrations substantially lower than their olfactory threshold. In this manner, construction-concealed sources of mould emissions can be detected not only by olfaction, but also by measurement of volatile analytes.

In this study the results of airborne MVOC measurements and air sampling for culturable fungi are presented from 131 residences in which mould odors of varying strength either were or were not detected.

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A MODEL FOR ASSESSING HEALTH RISKS ASSOCIATED WITH EXPOSURES TO LEGIONELLA BACTERIA

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ABSTRACT

Assessing the potential human health risks for developing disease caused by inhalation of Legionella bacteria depends on several factors. We developed an exposure assessment model based on a scenario in which *Legionella* bacteria are detected in a hospital cooling tower. Exposure risks were evaluated and compared for two potentially exposed individuals, a maintenance worker on the roof next to the cooling tower and a hospital patient inside the building. Variables for the emission source included bacterial concentration in the tower, tower water and air flow rates, and the percentage of tower drift. Pathway variables included distance from the tower, wind conditions, moisture level in the air, and air filters in the ventilation system. We determined that the risk of exposure to even one viable *Legionella* bacterium was 200,000 times greater for the maintenance worker than for the hospital patient. This exposure assessment model provides a useful approach for estimating health risks associated with exposures to *Legionella* bacteria.

KEY WORDS:

Legionella bacteria, exposure assessment, model, health risk

INTRODUCTION

The potential for inhalation of *Legionella* bacteria to cause serious disease in humans was dramatically demonstrated during an outbreak of a deadly pneumonia among American Legion conventioneers in Philadelphia, Pennsylvania in 1976 (Fraser et al. 1977). More than two hundred people became ill and thirty-four died, but the exact cause of the pneumonia remained unknown for months. The media quickly labeled the illness Legionnaires' disease and the newly-identified bacterial genus was subsequently named *Legionella*. *Legionella* bacteria were also later identified as the cause of a short-term, flu-like illness called Pontiac fever, named after a 1968 outbreak in that city.

During the course of several building investigations involving sources contaminated with *Legionella* bacteria, health concerns were expressed by both building managers and occupants regarding potential risks for developing illnesses due to airborne exposures to the bacteria from the contaminated source. The exposure assessment model presented in this report emerged as a result of these concerns and was developed to provide estimates for airborne exposures to *Legionella* bacteria under different scenarios. Variables incorporated into the model included not

only factors affecting the concentration of *Legionella* bacteria emitted from the source, but also the characteristics of the pathway to potentially exposed individuals and how the original source concentration was modified by that pathway.

MODEL DEVELOPMENT

The scenario used for developing this exposure assessment model was based on the discovery of *Legionella* bacteria in a hospital cooling tower. Cooling towers are one component of cooling systems commonly used in large buildings. In the absence of adequate anti-microbial treatment, *Legionella* bacteria can grow and proliferate in the warm water that circulates throughout these systems. Water containing *Legionella* bacteria may be released as an aerosol from a cooling tower and then be inhaled by exposed individuals. The model focused on estimating exposure risks for two individuals, a maintenance worker on the roof next to the cooling tower and a hospital patient inside the building. The model included results from a water sample collected from the cooling tower that indicated a concentration of 100 colony forming units of *Legionella* bacteria per milliliter of water (100 cfu/ml) in the cooling tower sump.

The model incorporated a variety of factors that could affect airborne concentrations of *Legionella* bacteria between the cooling tower source and the potentially-exposed individuals. Variables affecting the emission source concentration included the concentration of *Legionella* bacteria in the cooling tower water, water flow and air flow rates in the tower, and the percentage of tower drift. Tower drift is the percentage of the water aerosol that can escape from a cooling tower. Variables affecting the aerosol concentration in its pathway between the cooling tower and potentially-exposed individuals included dilution of the aerosol in the air based on distance from the tower and on wind conditions. Other pathway variables were moisture level in the air and the removal efficiency of the filters in the ventilation system. Moisture level, measured as relative humidity, was included because water evaporation can reduce bacterial viability by removing the protective water medium surrounding the bacteria in the aerosol droplets. The emission source and pathway variables for the model are summarized in the equation below:

$$\text{Exposure Concentration} = \frac{(\textit{Legionella}\ \text{Concentration})(\text{Tower Water Flow Rate})(\text{Tower Drift})(100\% - \text{HVAC Filter Efficiency}\%)}{\text{Tower Air Flow Rate}} \times (\text{Dilution in Air})(\text{Moisture in Air})$$

Exposure Concentration = colony forming units of *Legionella* bacteria per cubic foot of air.

Emission Variables: *Legionella* concentration = 100 colony forming units per milliliter of water. Tower water flow rate = gallons per minute. Tower air flow rate = cubic feet per minute. Tower drift = percentage of tower water flow rate

Pathway Variables: HVAC filter efficiency = percentage efficiency of filters in ventilation system. Dilution in atmosphere = estimated as 100:1. Moisture level in air = dry-down by atmosphere estimated as 100:1

The model also included the conservative assumption that inhalation of only one *Legionella* bacterium placed the exposed individual at risk of developing disease. The model purposefully

did not consider specific health factors, such as immunosuppression or other health conditions, on the likelihood of disease incidence. The model was used to estimate the time required to inhale one *Legionella* bacterium (exposure duration) for the two exposure scenarios.

RESULTS

Calculations using the model determined that the risk of exposure to even one viable *Legionella* bacterium was 200,000 times greater for a maintenance worker on the roof next to the cooling tower compared to a hospital patient in the building. Breathing at the same respiratory rates, the maintenance worker would inhale one bacterium after only 4.6 minutes whereas the hospital worker would have to breathe for 1.7 years before inhaling one *Legionella* bacterium. These results clearly demonstrate the value of evaluating variables that can alter the concentration of a bacterial aerosol emitted from a contaminated source. Such information can be useful when discussing the potential health risks related to exposures to *Legionella* bacteria with building managers and occupants.

CONCLUSIONS

Current estimates from the Centers for Disease Control and Prevention (CDC) and others project that 10,000 to 15,000 cases of Legionnaires' disease occur annually in the United States (Barbaree 1991; Millar et al. 1997). The number of individuals who develop Pontiac fever is likely larger and even more difficult to estimate. These figures are actually regarded as underestimates because infections caused by *Legionella* bacteria can only be conclusively identified when specific clinical tests for the presence of the bacteria are conducted. These estimates do, however, clearly demonstrate that exposures to *Legionella* bacteria and the illnesses that can result are current and important public health issues.

Development of an exposure assessment model that evaluates factors affecting the concentrations of aerosolized *Legionella* bacteria that may be inhaled by exposed individuals provides a useful approach for estimating the potential health risks for developing disease. Evaluation of these risks should include consideration of variables that affect both the emission source and the pathway to potentially-exposed individuals. The exposure assessment model presented in this report can be used to estimate differences in magnitude of exposure risk for individuals with different exposure profiles.

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CONCENTRATIONS OF VIABLE SPORES OF FUNGI AND ACTINOMYCETES IN VENTILATION CHANNELS

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ABSTRACT

In this work we studied if accumulation of fungal spores to the ventilation channels has any significance as microbial source in indoor air. Concentrations of viable spores were determined from samples that were collected from ventilation channels, and the geometric mean and median values were calculated. The concentrations of fungal spores and actinomycetes on ventilation channel surfaces were low even in abundantly dusty channels, and therefore accumulation of spores in the channels did not seem to form any significant microbial source in indoor air. In the samples from exhaust channels in buildings with verified mold damages, prevalence of fungal species such as *Acremonium sp., Aspergillus versicolor, Chaetomium sp., Eurotium herbariorum, Paecilomyces variotii, Phoma sp., Trichoderma viride* and *Ulocladium sp.* was better indicator for damages than the concentrations of fungal spores.

KEY WORDS:

fungal spores, actinomycetes, accumulation, ventilation channels, significance

INTRODUCTION

The ventilation systems form one possible route for spreading of micro-organisms inside the buildings. The ventilation ducts are usually made of metal plate, and when insulation is needed, it is placed outside of the ducts. Therefore, microbial growth on the channel wall surfaces is not likely to occur, unless water condensates in the channels (Nyman and Sandström 1991; Pasanen et al. 1993). Dust tends to accumulate to the channels, and it may contain microbes or their spores which originate from outdoor air, dusty filters in the ventilation machinery or abnormal microbial sources indoors. Aim of the present work was to study the concentrations and species of viable fungal spores on ventilation channel walls to determine if the accumulation of spores to the channels has any significance as a microbial source in indoor air.

MATERIALS AND METHODS

Altogether 68 samples from areas 100 cm^2 each inside the ventilation channels of office buildings were collected with a sterile swab. 54 samples were from supply channels and 14 from

exhaust channels; 24 of the supply channel samples and 12 of the exhaust channel samples were taken from buildings in which the occurrence of mold damages had been verified by analysis of material samples. The samples from supply channels were divided to four subgroups according to the amount of dust in the channels (amount of dust was determined by visual observation): clean (n = 7), moderately dusty (n = 16), and abundantly dusty (n = 9) channels, and channels in which the amount of dust was not determined (n = 22). The spores were extracted from the swab to 5 ml buffer solution (0.0003 M KH_2PO_4, 0.0021 M $MgSO_4$, 0.002 M NaOH, 0.02 % Tween 80). Dilutions from $1*10^{-1}$ to $1*10^{-3}$ were plated on 2% malt extract agar (MEA) and dichloran glycerol agar (DGA) for mesophilic fungi and tryptone-glucose-yeast-agar for actinomycetes. The plates were incubated at 25°C for 7 days (14 days for actinomycetes), after which the colonies were counted and the fungi were identified morphologically by using light microscopy to species/genus/type level. Geometric mean (GM) and median values were calculated from the spore concentrations with the Microsoft Excel™.

RESULTS

Spore concentrations

The GM and median values of viable fungal spores in ventilation channels are presented in Table I.

TABLE I: The GM and median values of viable fungal spores in ventilation channels.

	GM(cfu/cm²)		MEDIAN (cfu/cm²)	
Dustiness of channels	**MEA**	**DGA**	**MEA**	**DGA**
clean	0.19	0.14	0.1	0.1
moderately dusty	0.93	0.95	1	1
abundantly dusty	0.96	0.75	2	1
dustiness not determined	0.33	0.40	0.2	0.2
exhaust channels	1.28	1.27	2	1

Both the GM and median values of viable actinomycetes were 0.1 cfu/cm² in all of the subgroups.

Microbes indicating moisture damage

In the samples taken from supply channels, mold species such as *Acremonium* sp., *Aspergillus versicolor*, *Chaetomium* sp., *Eurotium herbariorum*, *Phoma* sp., *Paecilomyces variotii*, *Trichoderma viride* and *Ulocladium* sp., which have been regarded as indicators of moisture damages, were more often detected in dusty channels than in the clean ones, but their occurrence did not correlate with the existence of mold damages in the building. In the samples from exhaust channels these moisture damage indicator molds were almost solely found in samples from a building where severe moisture damages and mold growth had been detected.

DISCUSSION

Accumulation of fungal spores in the channels does not seem to form any significant microbial source in indoor air. The concentrations of fungal spores and actinomycetes on ventilation channel surfaces were low in all cases, even in the most dusty channels. This observation is in accordance with an earlier study by Pasanen et al. (1992). In the supply channels this could be expected because the supply air is filtered. We have analyzed some material samples which were taken from filters in air supply machines, and according to the results the fungal spores accumulate on the filters. If there are no leaks in the filter systems fungal spores are excluded from the air flow. Similar results have been presented earlier by Nyman and Sandström (1991). In just one of the buildings that were included in this study the ventilation air was recirculated, and the results from the two samples taken from ducts for recirculated air were not different from those obtained from samples from other supply channels with similar amount of dust.

In the samples from exhaust channels in buildings in which the occurrence of mold damages was verified by analysis of material samples, the prevalence of mold species such as *Acremonium* sp., *Aspergillus versicolor*, *Chaetomium* sp., *Eurotium herbariorum*, *Paecilomyces variotii*, *Phoma sp.*, *Trichoderma viride* and *Ulocladium* sp. was better indicator for damages than the concentrations of fungal spores. We have collected and analyzed air samples from almost all of the buildings that are included in the present study (data not presented here), and even if the spore concentration in indoor air was elevated, the concentrations in the exhaust channel walls were relatively low. In the ventilation of office buildings such air velocities in the ducts are used that most of the airborne fungal spores that enter the exhaust channels fail to settle out on the channel walls.

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FUNGI AND ENDOTOXIN EXPOSURE IN PAPER AND GLASS RECYCLING PLANTS

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ABSTRACT:

Personal sampling of microorganisms and total dust was carried out in plants receiving glass or paper for recycling. During manual sorting, the exposure to culturable fungi was significantly increased ($p=0.05$) at the glass recycling plants (105 cfu/m^3) compared to manual sorting of recyclable paper (104 cfu/m^3). The latter was at the same time significantly increased compared to sorting mail (103 cfu/m^3). Compared to other studies in the waste handling, the exposure to fungi during manual sorting of bottles was high and in general the exposure to endotoxin was at the same level or lower.

KEY WORDS:

paper, glass, bottles, recycling plant, mail sorting, sorting plant, recovery plant, recyclable materials, bioaerosol, fungi, endotoxin, glass particles.

INTRODUCTION

Exposure to microorganisms and health risk associated with work in the waste industry has come into focus during the last years. However, data are sparse on bioaerosol exposure in recycling plants processing glass/bottles and paper. As part of a large research programme (Midtgård & Poulsen, 1997), measurements of exposure to microorganisms and organic dust in bottle and paper recycling plants were performed. For comparison, measurements were carried out at mail sorting centers. In this paper, the exposure to viable fungi and endotoxin is reported. The clinical part of the study is presented seperately (Ebbehøj et al. 1998).

MATERIALS AND METHODS

INVESTIGATED PLANTS.

By the assistance of industrial experts, 12 recycling plants were selected from a total of 65 Danish waste recycling plants. The selected plants included 6 paper recycling plants, 4 glass recycling plants and 2 plants processing both paper and bottles. Two mail sorting centers were

included for comparison. Data from a paper recycling plant studied previously (Würtz & Breum, 1997) was also included.

The job function 'manual sorting' was widespread at all recycling plants as well as in the mail sorting centers. At large glass recycling plants, the job function 'manual sorting' included removal of broken glass and setting bottles on a conveyor belt leading to the automatic sorting line. In small plants, bottles were manually removed from a conveyor belt and placed in frames on pallets. At the paper recycling plants, 'manual sorting' included removal of irrelevant material from the conveyor belt. Usually this activity took place in ventilated sorting cabins.

All paper recycling plants but only one glass recycling plant had mechanical ventilation. At the mail sorting centers, 'manual sorting' included sorting of letters, small packages and magazines.

In addition to the manual sorting activities, all the plants and mail centers had other job functions including work in the reception area, driving frontloaders or forklifts, packing activities etc.

Bioaerosol sampling.

Personal bioaerosol sampling was performed from April to November 1996. Within each plant, sampling was usually carried out for 3 days with focus on the major job functions. An outdoor reference was included. Total particulate matter (TPM) was collected on cellulose nitrate/acetate filters and airborne microorganisms were collected on polycarbonate filters. Details about the sampling equipment and analysis has been described previously (Würtz & Breum,1997).

Microscopy.

By a combination of SEM (scanning electron microscopy) and light microscopy a few dust samples were analyzed for inorganic particles.

Statistical analysis.

Data are reported in terms of number of samples, median and range. Using SAS software, the Shapiro Wilk test (PROC UNIVARIATE NORMAL) and the Bartlett test (PROC DISCRIM) were applied for testing for normal distribution and variances for groups of data, respectively. Hypotheses on differences between groups of data were tested parametrically with one factor analysis of variance (PROC ANOVA). Data below the detection limit were set to half the limit in the calculations.

RESULTS

The exposure levels to endotoxin and culturable fungi are shown in Table I. Data for all job functions and 'manual sorting' are given.

For 'manual sorting' activities the exposure to culturable fungi differed significantly ($p<0.05$) among the three types of plants. The highest concentrations were found at the glass recycling plants. A similar tendency is seen for 'all job functions' (Table I). For glass and paper recycling

plants the dominating mesophilic fungi were *Penicillium* spp.. At the mail sorting centers no specific fungi dominated. The outdoor reference was dominated by *Cladosporium* spp.

TABLE I: Exposure to bioaerosols during handling of paper or bottles. Data are given in terms of medians and ranges, n = number of samples.

Working operation	**Moulds, 10^3 cfu/m^3 Median (Range)**	**Endotoxin, EU/m^3 Median (Range)**
Glass recycling plants		
Manual sorting (n=42)	121 (14-600)	5.4 (0.13-37)
All job functions (n=103)	64 (0.17-830)	6.7 (0.13-78)
Paper recycling plants		
Manual sorting (n=47)	15 (1.2-1300)	9.6 (1.2-100)
All job functions (n=99)	12 (0.26-1300)	8.5 (0.63-140)
Mail sorting centers		
Manual sorting (n=60)	0.92 (10.1-14)(26)	5.4 (1.2-20)
All job functions (n=66)	0.76 (10.1-14)(210)	5.4 (3.4-27)
Outdoor reference (n=36)	0.69 (10.1-22)(24)	1.5 (0.17-8.9)

[1]: ½ LOD; [2]: Number of samples at or below the limit of detection (LOD);.

Analysis by microscopy of airborne dust from glass recycling plants showed variable numbers of glass particles at a size which may become respirable. As seen in SEM, some of these glass particles were characterized by razor-sharp edges.

In general the endotoxin exposure at the plants was low and with few exceptions slightly above the background level.

DISCUSSION

The exposure to culturable fungi during manual sorting of bottles was high and at a level comparable to data reported on waste collection of household waste (Nielsen et al. 1997). Furthermore, the concentration in glass recycling plants exceeded the exposure level in a Swedish plant when sorting the dry fraction of household waste using similar sampling technique (de Davila 1997).

The glass recycling plants were except for one characterized by lack of mechanical ventilation. The floors and sorting lines in these plants were wet which may favor growth and sporulation of molds such as *Penicillium* spp., which were the dominating fungi. In this context the supply of organic matter from residues in the bottles, jars and cans is also important. The high exposure levels at the sorting plants may have been caused by damp environments, intense manual sorting activities as well as the general low occupational hygiene standards.

Exposure to molds at levels of 10^6 spores/m^3 is associated with respiratory symptoms, mucous membrane irritation and ODTS-like symptoms in saw mill workers (Eduard et al.1993) and chronic exposure to higher levels (10^8 - 10^9 spores/m^3) has been reported to cause allergic alveolitis in farmers (Malmberg et al.1988). In the present study the viable counts of fungi at glass recycling plants ranged from 10^4 to 10^5 cfu/m^3 and total amounts of microorganisms some-

times reached the level of 10^6 cells/m^3 (data not shown). It is therefore likely that work at the glass recycling plants can be associated with an increased risk of respiratory symptoms. This is supported by data from the matching questionnaire study which showed significantly increased complaints of dyspnoea among employees at glass recycling plants (Ebbehøj et al. 1998).

Although exposure to molds are believed to be strongly implicated in this symptom, it is not obvious whether other biologically active compounds and the presence of fine airborne glass particles may have contributed. Perhaps the razorsharp glass particles may cause an irritative effect in the bronchia and alveoli. This effect may enhance entrance of inflammatory or allergenic compounds present in the organic fraction of the airborne dust.

In contrast to viable fungi, the exposure to endotoxin was low both for the glass and paper recycling workers. Only few samples exceeded the recently proposed health based occupational exposure limit of endotoxin at 50 EU/m^3 (Heederik and Douwes 1997).

CONCLUSION

This study showed that exposure to culturable fungi was higher at the glass recycling plants compared to the paper recycling plants. In manual sorting of recyclable bottles, the exposure to fungi was significantly above the level in manual sorting of recyclable paper which again was significantly above the level in manual sorting of mail. The airborne indoor concentration of fungi at glass recycling plants was comparable to the airborne outdoor concentration of fungi when collecting household waste.

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AIR QUALITY RESTORATION IN A FUNGAL CONTAMINATED BUILDING; A CASE STUDY

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ABSTRACT:

Attempts to mitigate microbial contamination of a four story office building by use of common standard cleaning procedures proved inefficient by post abatement analytical results.

Toxigenic, allergenic and pathogenic fungi were identified in sampling evaluation as a follow up to microbial abatement. Microorganisms such as *Stachybotrys*, *Aspergillus*, *Penicillium*, *Cladosporium* and *Fusarium* were detected in air and surface samples of a four story office building. Review of the chain of events revealed that microbial recolonization, cross-contamination and recontamination had occurred at this facility.

IED, Inc. an environmental company specializing in microbial abatement and air quality restoration in indoor environments was commissioned to the project of biocidal clean-up and air quality restoration. Success of such projects are strictly dependent on applied scientific knowledge and experience. IED, Inc. successfully completed the biocidal and air quality restoration project as proven by post abatement analytical results performed by an independent third party monitoring company and laboratories.

KEYWORDS:

Stachybotrys, *Aspergillus*, *Penicillium*, *Cladosporium*, *Fusarium*, Sporicide, Organic Biocide, Chlorine Dioxide, Hypochlorites, Quartenary Ammonium Compounds.

INTRODUCTION:

Building vacancy, flooding, water leaks and lack of maintenance caused sever fungal contamination of four story office building. Review of reports issued by environmental companies strongly indicated that the indoors were severely contaminated by hazardous to health fungi such as *Stachybotrys*, *Aspergillus*, *Penicillium*, *Fusarium* and other toxigenic, allergenic and pathogenic microorganism, especially on topical surfaces and air conveyance system.

Review of the chain of events (testing and decontamination, etc.) reveals that microbial recolonization, cross-contamination and recontamination had occurred at this facility. That may have been caused by improper abatement technology, use of improper biocides, or improper concentration of biocides, which could have been avoided if the abatement process was professionally planned, designed and performed.

MATERIALS AND METHODS:

The testing protocol used to determine the success of the project involved over 300 pre and post abatement test samples. Tests were conducted throughout the above building, both outside and on all four floors. The sampling methods employed were specifically designed to find *Stachybotrys* presence, in addition to the more common fungi such as *Penicillium*, *Aspergillus* and Fusarium, previously identified.

Sampling of surfaces and air were also taken to collect evidence of non-viable organisms. Airborne Sampling was conducted using an Anderson N6 single stage sample operated at a flow of 28.3 L/minute. The sampler was calibrated using an in line rotameter calibrated with a Gilibrator bubble meter. Media samples used were 2% MEA and cornmeal agar (CMA). A full respiratory and body protection program was implemented for the abatement workers, including a full-faced powered air purifying respirator with HEPA filters and disposable full-body coveralls, head covers, gloves and boot type covers. Vinyl Acetate Adherence Surface Collection, and Burkard Trap Air Sample Collection were used to gather evidence of airborne or surface contamination from any remaining non-viable *Stachybotrys* spores. The samples were examined to determine the presence and concentration of any organisms found.

The monitoring involved sampling of topic air, surfaces, air conveyance systems, carpeting, elevator shaft and lobbies before, during and after abatement completion.

Airborne particulate levels were monitored via nephelometric laser scatter measurements, to provide a real time indication of airborne release situations as they occur. Pressure differentials between the occupied and work areas were monitored to confirm that the occupied areas were positively pressurized relative to the work areas and thus prevent the flow of contaminants into the occupied area.

Pressure differentials between the occupied and abatement areas were monitored as well to confirm that the occupied areas were positively pressurized relatively to the abatement areas (minimum of 0.02 inches of water gauge). A minimum of four air changes per hour in the work area was maintained throughout the abatement. Following is the General Cleaning/Disinfection protocol as used in this project:

1. Isolation of Work Areas via NJAC 5.23-8 guideline airflow control techniques, including protection of the third floor intake plenum bypass and common areas.
2. Removal of large items and debris (loose and or dusty materials) from the work area.
3. Cleaning and disinfection of associated roof mounted air handling units (AHU).
4. HEPA vacuuming and wet wiping of top and upper surfaces (decks, beams, tops of ducts, etc.) using a solution of sodium hypochlorite (1:10).
5. Cleaning and disinfection of ductwork where accessible at duct openings, and at penetrations for biocide application.
6. HEPA vacuuming and wet wiping of walls, ledges and other mid-height surfaces mixed with diluted alcohol.
7. HEPA vacuuming and wet wiping of floors.

8. Application of biocides such as Hypochlorites and Quaternary Ammonium for sufficient biocidal time.

The Overall Procedures for Surface Cleaning and Disinfection:

(Performed once numbers 1. and 2. above have been performed.)

1. HEPA vacuum and waiting at least one hour for the dust to settle.
2. Wet wiping with appropriate biocidal solution, brushing of surfaces as required.
3. Application of biocidal solution such as to keep it wet long enough to provide biocidal action.

The Overall Disinfectant Protocol for Each Surface, specifically was as follows:

1. Metals: organic biocide with surfactant. Biocidal time of at least 30 minutes. (Not for use within HVAC components.)
2. HVAC Interior Components: Chlorine dioxide solution. Biocidal time of at least 20 minutes.
3. Other Surfaces: Biocides and surfactant solution. Biocidal time of at least 30 minutes. Two very distinct groups of Biocides, specifically Sporicides were used with alternations and specific contact time. (1) Hypochlorites and (2) Quaternary Ammonium Compounds:

   Hypochlorites possess sporicidal properties, when used in appropriate concentration and biocidal contact time, but their activity is greatly reduced in the presence of organic matter. They are more active at acid than alkaline pH, the active moiety being HClO.

   The mode of action of Quaternary Ammonium Compounds is believed to be associated with the agents effect on the cytoplasmic membrane, which controls cell permeability. When mixed with other chemicals, such as low concentrations of alcohol, certain sporicidal features are enhanced. Being proprietary formulation, no further details can be provided.

4. Chemical Neutralizer for surfaces treated with biocides/surfactants to eliminate any chemical residues originating from Biocides and their by-products.

   The above remediation procedures were followed by the on-site IED personnel. The disinfectant wetted waste was disposed of in double layer 6 mil polyethylene containment, as construction debris.

RESULTS:

Sampling and evaluation performed prior to the fungal abatement showed high concentrations of *Stachybotrys chartarum* (1.4 to 1.8 million colony forming units per square inch of surface area). *Stachybotrys* was not detected in any of the outdoor samples. This fact was indicative that an interior source or reservoir might exist. Other allergenic and toxigenic fungi were identified in quiescent sampling and included *Cladosporium*, a common outdoor taxon, and *Penicillium*, *Aspergillus*, *Fusarium* and *Alternaria* which are indicative to outdoor taxa penetrating the building indoors.

Surface sampling resulted in identification of Aureobasidium pullulans in the northwest quadrant sample and *Fusarium* - a fungal group containing toxigenic species, and were dominant in

the southwest quadrant sample. Prior to the abatement dust was observed on the concrete floor and other horizontal surfaces. Airborne dust was monitored and resulted in significant high concentration. It should be stressed that dust is a reservoir, amplifier and carrier of microbial contaminants.

Based upon test results from laboratories, the abatement project has successfully removed all *Stachybotrys* from the building. Upon the conclusion of all remedial work all other microorganisms were at or below normal outdoor levels.

TABLE I: Results of Postabatement Air Sampling in CFU/M^3 per floor

Floor	Aspergillus	Alternaria	Cladosporium	Fusarium	Penicillium	Stachybotrys
IV	3	0	0	2	1	0
III	0	6	2	0	1	0
II	1	3	3	1	5	0
I	15	5	20	15	26	0
Outdoor Air:	123	45	406	38	96	0

TABLE II: Postabatement Surface Sampling in CFU/CM^2

Floor	Aspergillus	Aureobasidium	Cladosporium	Fusarium	Penicillium	Stachybotrys
IV	6	0	10	8	11	0
III	4	6	5	7	18	0
II	10	2	8	3	13	0
I	18	22	3	5	14	0
Outdoor	$1.1x10^6$	$1.2x10^6$	$1.0x10^6$	$1.2x10^6$	$1.6x10^6$	0

DISCUSSION:

Stachybotrys is found in the outdoor environment. It is found inside when air currents or occupant traffic carry it in. When conditions are not conducive to growth (too dry, for example) the spores do not grow into colonies, remaining dormant. Any building can have *Stachybotrys* in it. A problem only arises when the conditions within the building are conducive to the growth of colonies.

Stachybotrys is a toxigenic fungus which can inhabit wet, cellulose containing materials. Such materials existed at the above site, due to past water damage from roof and curtain wall leaks. The water had moistened areas of sheet rock, which allowed the fungus to grow in the wet cellulose containing materials such as paper.

The fungus reproduces via spores, which can be transmitted by air currents. When these spores land in a place conducive to growth, new colonies are formed. These colonies reproduce, and create more spores, repeating the cycle.

Water damaged areas, when dried, will no longer support active growth, as the fungus requires a moist environment. Other organisms, those requiring less moisture to thrive, can then replace the previous population of *Stachybotrys*.

The toxic component of *Stachybotrys* is primarily in the organism's exterior membrane. This means that the dead organisms are just as toxic as live ones. This is why tests were conducted

for viable, as well as non-viable *Stachybotrys* presence, and specific concentrations of biocide was used.

Other organisms found in the indoor environment were present, also carried in from the outside. As outdoor air will always be drawn in, to provide occupants with fresh air, organisms found outside will always be drawn inside.

Abatement, therefore, cannot realistically be expected to result in this building remaining free from microorganisms. The goal is to prevent the building from developing conditions conducive to microbial growth. When outdoor organisms are drawn in, they are eventually taken back out by the exhaust system, or they remain dormant within the building.

These dormant organisms are time bombs waiting to go off when water damage, excessive humidity, or other factors conducive to their growth occurs. It is not feasible to continually remove them. It is feasible to continually maintain the building in a non-growth conducive manner. Much microbial growth occurs before the growth becomes visible. Often, the growth is never visible, and airborne levels are dangerously elevated with no observable clues.

CONCLUSIONS:

1. Moisture accumulation in wall cavities, walls and elevator shaft, and chronic neglect was the source of primary fungal contamination in the entire building.
2. Review of the chain of events such as previous decontaminations and laboratory results, revealed that fungal recolonization, cross-contamination and recontamination had occurred. That may have been caused by poor design and poor abatement performance and use of inadequate biocides.
3. Use of general contractors and improper abatement technology without experience in disinfection or decontamination led to problems resulting in fungal recolonization and recontamination.
4. The stringent remedial protocol employed by IED successfully eliminated the fungal contamination, by using a variety of biocides to match the variety of identified fungi.
5. Prevention is the best cause of action and includes moisture prevention, adequate air filtration, mechanical hygiene and appropriate maintenance. However, if for any reason, a build-up of microbes does occur, immediate professional treatment is called for.

SAMPLING, RESULTS & REMEDIATION IN 300 "SICK HOUSES"

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ABSTRACT

Inhabitants in "sick house syndrome" (SHS) homes suffer from allergy, asthma, sinus and other respiratory problems, including hypersensitivity pneumonitis and aspergillosis. Reports from 300 SHS homes were compared to randomly-selected reports from 150 homes inspected as part of pre-purchase agreements in the Boston area. SHS homes were almost twice as likely as other homes to have forced hot air heat, central air conditioning and finished/carpeted basements. Elevated levels of mold were found in 74% of SHS homes. The largest sources of bioaerosol were found to be carpeting; heating, ventilation, and air conditioning (HVAC) equipment; and beds and sofas. Respirable "carpet dander" from damaged wool carpet fibers can be an irritant. Effective SHS remediation may include: carpet removal; thorough coil /duct/blower cleaning along with replacement of contaminated fiberglass lining materials in HVAC equipment, and cleaning fleecy items with dry (super-heated) steam. Mite-barrier mattress and pillow covers are always recommended. SHS may be a cause for increased asthma rates.

KEY WORDS:

Sick house syndrome (SHS), asthma, allergy, bioaerosol, house dust mite, steam cleaning, carpet, carpet dander, duct cleaning, air conditioning.

INTRODUCTION:

Since 1939, we have conducted hundreds of "home environmental surveys" to help families with air quality problems, and thousands of pre-purchase inspections for home buyers. On a "home environmental survey," we obtain the history of the problem in an interview, then take air, dust and other bulk dust samples. In some homes, clients have had chronic symptoms for years. In other homes, individuals call after becoming ill just after moving, or soon after turning on the heating or cooling; or after having basement moisture problems. Complaints include wheezing, allergy symptoms, coughing, and others.

We reviewed our reports from 300 "sick house syndrome" (SHS) homes and looked at building system characteristics, observations and air sampling results. For comparison, we also randomly reviewed 150 of our pre-purchase, home inspection reports, for the presence of forced hot air heat, central air conditioning, finished/carpeted basements and moisture stains. Note that on a typical pre-purchase home inspection, no air or dust sampling is undertaken.

MATERIALS AND METHODS:

A blade containing a notched edge, 50 micron deep by 1 cm long, is prepared by rubbing a single-edge razor or drywall knife blade on a 1 cm strip of #400 grit carbide paper adhered to a flat surface. The blade, drawn at an angle, is used to spread a thin film of Dow Corning high vacuum (silicone) grease onto the center of a standard 1 x 3 inch (approximately 25 x 75 mm) microscope slide. For particulate sampling, greased slides are placed in a Burkard Personal Volumetric Air Sampler (10 liters/minute flow rate, usually for 3 minutes) or an Allergenco Samplair MK-1 (15 liters per minute flow rate, either for 5 or 10 minute "on" time and 5 or 10 minute "off" time). Generally, one Burkard air sample is taken first in an undisturbed room and then a second air sample is taken in a "disturbed" room, agitated by either waving a book to aerosolize dusts, or by rubbing the carpet if present. "Pat" samples from cushioned surfaces are obtained by tapping the surface and taking a several second Burkard sample in the resulting "dust cloud." Acid fuchsin (.1% in lactic acid) is used to stain samples for microscopy. All samples are viewed at 400 X; some at 1000 X with oil immersion.

Viable mold samples are obtained with an Andersen N-6 (30 liters/minute flow rate; 1, 2 or 3 minute sample times are used, depending on the "dustiness" of environment) and Petri dishes containing either Sabouraud or malt extract agar. Samples are incubated at either room temperature or 25°C.

Dust samples are obtained using clear, 12 mm sticky tape; for transportation, tapes are lightly adhered to a small polyethylene bag and placed in a second, larger bag. For microscopic examination, a tape sample, cut into a 1 cm strip, is placed dust -side facing up on a drop of 85% lactic acid (for adhesion) on a glass slide. Acid fuchsin stain and a cover slip are added.

The surface of air conditioning coil is sampled with sticky tape at the "upwind" side only, where dust accumulates. Fiberglass material is also sampled with sticky tap, usually at the bottom of the blower cabinet and at areas where moisture staining is noted.

For quantitative immunoassay of house dust mite and dog allergens, dust samples were sent to ALK Labs (Spring Mills, PA); for qualitative mite analysis, dust samples are stained with acid Fuchsin and observed with a microscope for mite fecal pellets and body parts.

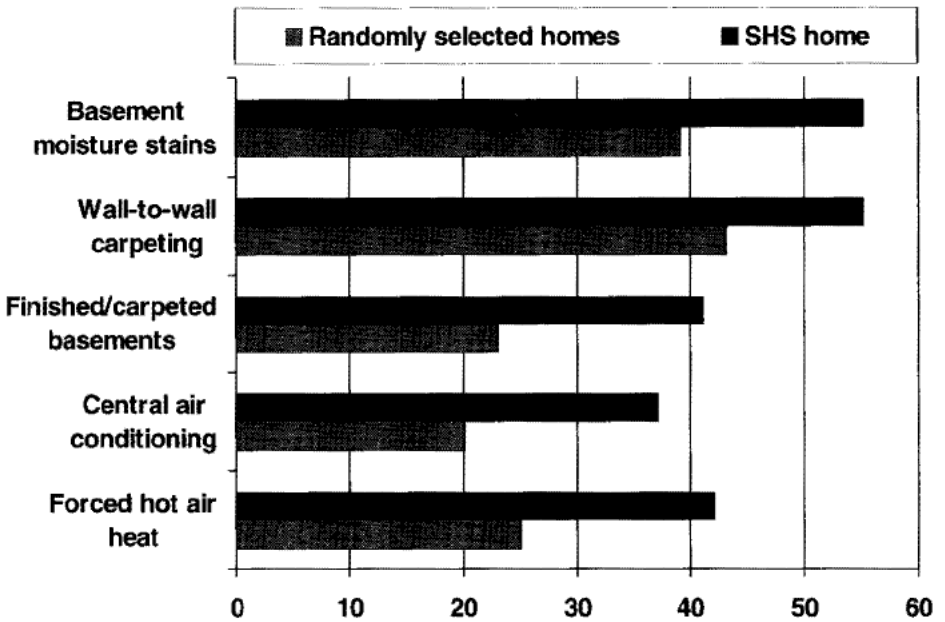
A Fogacci steamer (Fogacci, Bologna, Italy) provided "dry" (superheated) steam.

RESULTS:

In the SHS homes, about 20% of inhabitants suffered from asthma, 80% had allergies and/or suffered from respiratory complaints and miscellaneous symptoms/illnesses such as nasal/sinus congestion, chronic fatigue, multiple chemical sensitivity, hypersensitivity pneumonitis (3 cases), and aspergillosis (one case).

As illustrated in Table I, in a SHS home, forced hot air heat, central air conditioning and finished/carpeted basement are almost twice as likely to occur than in a randomly-chosen house. Wall-to-wall carpeting (in 55% of SHS homes versus 43% in randomly chosen houses) and basement moisture staining correlate less positively.

Table I: Comparison of Randomly -Selected Homes and SHS Homes



The results of air and bulk sampling in SHS homes corroborate these findings. Most SHS homes have elevated levels of airborne fungal spores. Whereas spores in air samples from typical homes are mostly individual or in small groups, SHS air samples often contain long chains of *Penicillium* or *Aspergillus* conidia, or clusters of conidial chains. Although all dust samples contain a few spores, (most often a variety), surfaces and dusts where fungi are actually growing have hyphal networks and masses of "conidia", often in characteristic clusters or on conidiophores. Bulk sampling on sticky tape is the optimum way to distinguish such surface growth from random accumulations of spores; swab sampling followed by culturing, though useful for speciation, may not necessarily distinguish between the simple presence of settled spores versus active growth of fungi in the substrate.

Almost 100% of fiberglass lining materials and air conditioning coils have hyphal masses and conidia, suggesting active mold growth at some location; over 50% of blower cabinets and blowers have mold growing on them. The most likely locations for growth are the coil surfaces (at the incoming side) where dusts are dampened by condensate, and the fiberglass lining materials in the immediate vicinity of the coil, where air leaves the coil at its dew-point (100% humidity) and may carry droplets of entrained water. Fiberglass lining materials that have been wet by condensed water from cold piping to coils or clogged drip trays are also common locations for fungal growth. Dusts stuck on metal blower cabinet walls and bottoms in contact with concrete surfaces are often colonized as well.

In HVAC systems, the only visible source of nutrients for mold growth is house dust. Often, starch granules, from use of body powders, constitute a significant proportion of the

biodegradable dust. The most commonly observed genera in SHS home air samples are *Penicillium, Aspergillus* and *Cladosporium.*

In one forced hot air heating system, a sample from the 2 cm dust layer in the return duct contained dust mites and 20 micrograms per gram (μg/g) house dust mite allergens. Basement carpets and fleecy furniture are more common sources of house dust mite allergens. The highest level of mite allergens observed during our sampling, 200 μg/g, was found in a basement playroom couch. Steam ("dry," i.e. superheated steam with no liquid water) was reported by M.J. Colloff et al. 1995 (1) to be an effective means to kill mites and reduce mite allergens in carpeting. Superheated steam was used to treat a sofa cushion containing 35 μg/g dog allergen, and 30 μg/g dust mite allergens. Repeated applications of dry steam reduced levels of both dog and dust mite allergens to less than 1 μg/g. A similar reduction in mite allergens was observed after steaming a mattress.

In SHS homes, 74% had either or both elevated airborne spore-levels (Table II) and visible mold growth; 33% had evidence for mite infestation; 22% contained airborne pet dander.

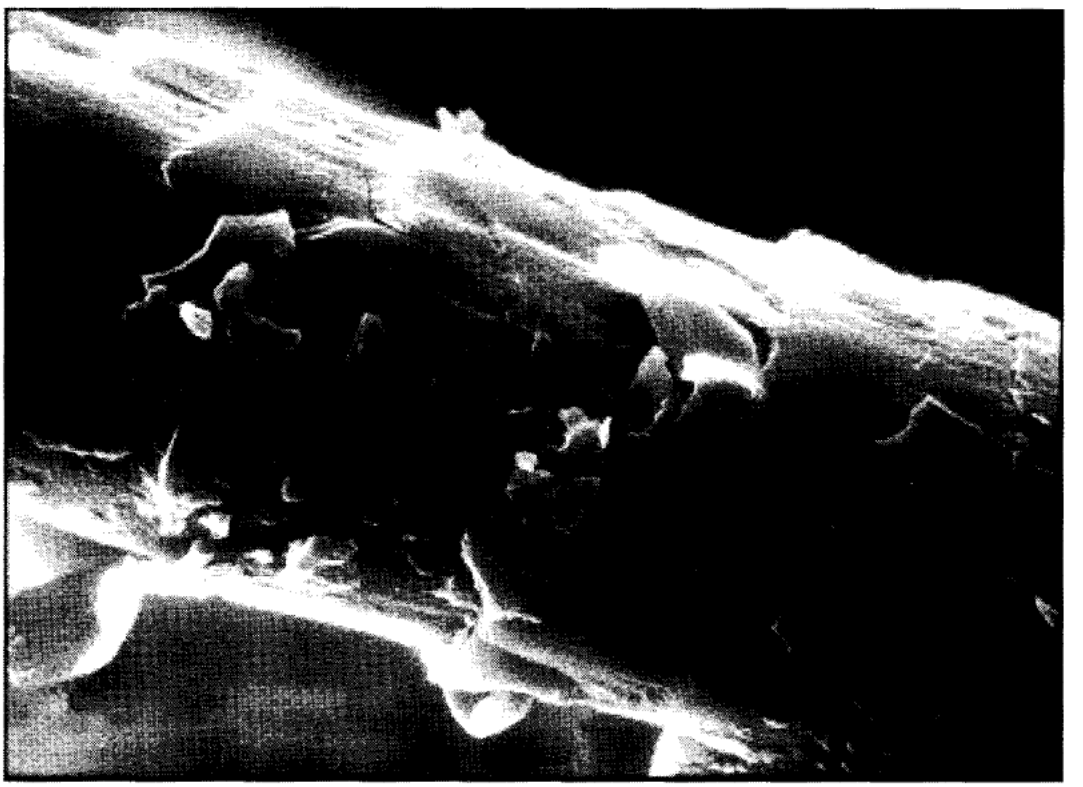
TABLE II: Examples of Symptoms and Molds Found in SHS homes

Client	Symptoms	Sample Location	Andersen CFU/m³	Burkard spores/m	Other
Child	Asthma; allergy	Basement:	11,700	200,000*	*Cladosporium
(hot air heat	(pollen, mite)	Den:	No viable	1600	(moldy furniture in wet
basement	chronic fever	Vent: blower on	Not tested	300,000	basement)
return)		Exterior	690	6600	
Male adult and	coughing; scratchy throat	Bedroom:		1500	Penicillium/ Aspergillus
wife	asthma	Basement:		6000	same
Adult female	pneumonia, bronchitis after moving in	Basement: (door left open for cat access to box.)		15,000	Penicillium/ Aspergillus
		Living room:		13,000	Pen/Asp
		Exterior		700	Cladosporium
Male adult	asthma	Basement:		6000	(carpeted)
		Den		233	(wood floor):
Male	asthma as child	Finished basement (with carpet)	6000		Asthma returned after moving in.
10 year-old boy	headaches	Living room:		4500	Penicillium/
(missed about	(reduced	(carpeted)			Aspergillus-type
1/3 school days)	symptoms at aunt's house)	Bedroom: (no carpet)		2500	(dander 2000+ per cubic meter)
Adult female	chronic fatigue	Dining room:		1700	Mostly brown spores.
(works at home;	eczema, dermatitis	Dining room:		6000	(Periconia?)
hot air furnace)		(agitated carpet)			
Father and son	asthma (both)	Kitchen air:		2500	
(basement office	Refrigerator vent:		5500		
with carpet.)		Basement office:		2700	(quiescent)
		Basement office:		7000	(after walking)
Female adult	post nasal drip, congestion,	Living room:		1500	Symptoms worst in bedroom:
	eye irritation	Bedroom		20,000	(Pen/Asp)

In one Burkard air sample, wool carpeting contributed about 30,000 particles per cubic meter of "carpet dander," most respirable, varying in size from 2 microns to 50 microns. The scanning electron micrograph (SEM) in Figure I depicts friable wool cuticle, and the SEM in Figure II illustrates a friable wool cortex.

FIGURE I: Deteriorated wool fiber cuticle, SEM (2000x). Cuticle particulates, about 30,000 /m^3, many respirable, were observed in an air sample in the carpeted room.

FIGURE II: Friable wool cortex "strands," SEM, (1000x). Cortex strands are fully exposed where the cuticle has fallen away from wool fiber. Fragments of cortex "strands" may appear as respirable particulates in air samples.



DISCUSSION:

Any equipment that distributes air can accumulate biodegradable house dust and become a source of bioaerosol if moisture or elevated humidity is present; sources include soiled cooling equipment (including window A/C units), blowers, ducts (especially in cold attics or damp crawlspaces), dryers, refrigerator drip trays, humidifiers and dehumidifiers, etc. Staining may indicate past presence of bulk water, but high relative humidity and dew-point condensation are important factors in mold and insect colonization in basements, ducts, carpeting, fleecy surfaces and beds. When these colonized surfaces are subject to disturbances, bioaerosol is produced.

Friable hairs from wool carpeting can be a source of bioaerosol from both the cuticle and the cortex of the hair. Most of the respirable particulates originate in the cuticle, though in some cases, large numbers of respirable fibrous particulates from the cortex may predominate. The presence of these bioaerosol particulates may be associated with coughing and eye irritation.

Wool cuticle and cortex bioaerosol particulates can only be detected by sampling and microscopy. In many problem homes, other irritating and allergenic aerosols cannot be detected by odor or any means other than sampling. Under some circumstances, even extensive mold and mite colonization cannot be readily observed. Sampling and microscopy can be used very effectively to identify contaminated materials and to determine the extent of a problem.

Recommended SHS remediation includes: removal of wall-to-wall carpeting; thorough cleaning of hot air heat and central air conditioning systems (replacement of fiberglass lining materials, thorough coil /duct/blower cleaning, installation of 40%-efficient media-filters to prevent future dust accumulations); removal of house dust, especially from heat distribution components (radiators, convectors, etc.); use of HEPA-filtered vacuums; installation of allergen-control covers over mattresses and pillows; elimination of down bedding; removal of water sources in basements; improvements to grading and drainage at the exterior of the house. In homes of highly sensitized individuals, remediation of very contaminated basements has been undertaken with asbestos-level precautions.

CONCLUSIONS:

Elevated levels of bioaerosol from mold, insect allergens, pets, and wool carpeting are associated with SHS homes. SHS homes were almost twice as likely as other homes to have forced hot air heat, central air conditioning and finished/carpeted basements. Fungal proliferation on soiled A/C surfaces due to inadequate filtration is a major contributor to SHS. Complete remediation of dusts can eliminate most SHS symptoms. It is probable that a significant proportion of the increase in asthma over the last 15 years is associated with the increased use of furnaces, heat pumps and central air conditioning; and the increased use of below-grade, finished spaces for work by adults (home offices, retirement) and for play by children at home and in daycare.

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IDENTIFYING AND PREVENTING FUNGAL CONTAMINATION PROBLEMS IN NEW HOME CONSTRUCTION

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ABSTRACT

The objectives of this study were to identify construction materials and practices prone to introduce fungal contamination into new single family homes, and to identify preventive maintenance procedures which limit fungal colonization and amplification.

This study was performed over a two year period in North Carolina. Eight construction sites were observed during construction and during the first year of occupancy.

Building materials were improperly stored and exposed to rain and/or high humidity. Inadequate drainage caused many crawl spaces to remain damp. Many HVAC systems were inadequately sealed, enabling rainwater and construction debris to enter the systems, and thereby providing suitable fungal substrates. Negative pressure within the building envelopes caused air infiltration from the crawlspaces.

Many of the observed problems found could be avoided by educating builders and homeowners. Early identification and prevention of potential fungal reservoirs and amplification sites is preferable to costly analysis and repairs afterwards.

KEYWORDS

Fungal contamination, preventing fungal contamination, indoor air pollution, moisture control.

INTRODUCTION

The objectives of this study were to identify construction materials and practices prone to introduce fungal contamination into new single family homes, and to identify preventive maintenance procedures which limit fungal colonization and amplification.

Fungal spores are found in virtually every home and building. Generally, the composition of the air spora is a mixture of species which resembles that which is found in outdoor air. The indoor counts may be lower depending on ventilation and air-conditioning practices. High level of fungal spores and atypical mycoflora in indoor air is most often a sign of moisture or water damage. In recent years several studies have confirmed strong associations between adverse health effects and indoor fungal exposures (Pasanen, 1992; Hyvärinen et al. 1993; Flannigan and Miller,1994).

Fungi are able to grow and proliferate on a wide range of organic materials. Fungal growth is influenced by various environmental factors. According to several studies, the most important

factor controlling fungal growth is the moisture content of the substrate. The surface relative humidity is a complex function of building material moisture content, material properties, and local temperature and humidity conditions (Pasanen et al. 1992b; ASHRAE, 1997).

Most common reasons for moisture damage and dampness problems in buildings are water leaks, defective drainage, inadequate ventilation and moisture condensation resulting from faulty thermal insulation. Particularly, heating, cooling and ventilation (HVAC) systems and ductwork have been considered major contributors to moisture problems (Christian et al. 1991; Hyvärinen et al. 1993).

MATERIALS AND METHODS

This study was performed over a two-year period in 1996 - 1998 in Cary, North Carolina. Eight houses under construction (#1 - #8) were selected for the case study. The houses represent the range of construction types for medium priced single family houses built in North Carolina. Each house has two stories, a crawlspace, an attached garage, and two separate forced-air HVAC systems. The climate in North Carolina is a cooling dominated mixed climate.

The construction sites and practices were observed during construction 1996-1997. The completed houses were observed during the first year of occupancy 1997-1998. Evaluation of site conditions was based on visual observations. Photographs were taken, and bulk samples were collected of major problem areas detected. Samples were processed and analyzed according to accepted mycological methods.

RESULTS

STORAGE AND HANDLING OF CONSTRUCTION MATERIALS

On all construction sites the building materials were found to be improperly stored and exposed to rain and/or high humidity. On all sites the wood used for framing was lying on mud. The mud covered wood had visible fungal colonies, and it was not cleaned before or after framing. On site #4 approximately 60% - 80% of the frame had turned almost black due to fungal growth. On site #1 heavy storm winds had scattered a stack of rigid insulation panels onto the street. The panels which had been laying in muddy puddles were later installed into the house.

HEATING, COOLING, AND VENTILATION

On site #3 the attic air-conditioning unit was missing both the main and backup drains. The condensate drained into the attic insulation and into the upstairs ceiling for the first two months of the high humidity season. The builder eventually installed the drain and painted the ceiling, but did not replace any of the defected construction materials (e.g., fiberglass insulation and plywood).

On every site significant amounts of water condensed on the air distribution box in the crawlspace during the high humidity season. The flexible duct insulation and the ground under the distribution box were constantly wet.

On sites #1-#2 the HVAC systems were left unsealed. On sites #3-#5 and #7-#8 the HVAC systems were poorly or only partially sealed. During the first year of occupancy dirt, sawdust, insulation fibers and other contaminants entered the systems when they were operating. Furthermore, on sites #1 and #2 rainwater entered the systems causing visible fungal growth in the ductwork. On site #1 the water leaked into the air-conditioning unit through the air return duct collar attached to the unit. The predominant fungi identified inside the duct collar were species of *Penicillium*, *Cladosporium*, *Aureobasidium*, *Ulocladium*, and yeasts. Also *Bacillus* overgrowth was detected.

Supply air duct leaks in the crawlspace and in the attic caused depressurization in houses #1-#5 and #7-#8. Due to the negative pressure the outside air infiltrated the building envelopes mainly through unsealed floor joints, and openings on walls for plumbing and electric outlets. This infiltration caused houses #1-#3 and #7 to have strong crawl space odors. In houses #2 and #3 the odor of fungal VOCs was predominant. Houses #1 and #7 had an intense earthy odor which is often associated with soil bacteria (Nevalainen et al. 1990). The predominant species of bacteria identified inside house #1 were Bacillus and actinomycetes.

The HVAC system of house #1 was eventually sealed. This revealed that the boots for the first floor air supply registers were inadequately insulated. Until the boots were sealed the leaking air conditioned the one foot tall space between the floor and thermal insulation below the floor. After the entire ductwork was sealed the temperature of the uninsulated boots was significantly below the dew point in the space. Water condensed on the boots causing the wooden subfloor to be constantly wet within a one-foot radius around each boot. The subfloor was covered with visible fungal colonies within two months after sealing the ductwork. Also, wherever the flexible air ducts touched the thermal insulation the duct and the insulation were constantly wet.

In house #1 the subfloor around the boots for living room and bathroom air supply registers remained wet after insulation was applied. The interior floor material was hardwood or thin vinyl above the problem areas. The metal registers resting on the floor became very cold when the air-conditioning was operating. The thermal conductivity of hardwood and thin vinyl provided a thermal bridge from the registers to the subfloor. The condensation did not stop until a weather strip was placed under each register.

The casings and duct connections of all bathroom exhaust fans were unsealed, allowing humid bathroom air to escape inside the bathroom ceiling. House #1 had a kitchen exhaust hood ventilated to outdoors. The exhaust ductwork was not sealed, allowing cooking vapors to escape inside the kitchen ceiling and the wall. All dryer exhaust ducts were inadequately sealed allowing lint and hot humid air to escape inside the utility room walls.

Drainage around the foundation

Due to the lack of proper drainage around the foundation, crawl spaces on sites #1-#3 and #5-#7 remained very damp. Crawl spaces on sites #2 and #3 had big puddles of water that housed several frogs. In the crawlspace of house #5 big mushrooms were found. These problem sites lacked sufficient positive grading or extenders attached to the downspouts to carry the rainwater away from the foundation. On site #1 the problem was later corrected by par-

tially re-grading the lot, and attaching underground pipes to downspouts to carry the rainwater away. On site #6 the problem was partially corrected by re-grading the lot.

Construction debris

On all sites the floors were swept regularly. However, large amounts of sawdust and wood chips were left inside the walls.

All HVAC systems were operating during the construction, causing large amounts of construction dust to enter the systems. Also large amounts of construction debris fell into the ductwork through open floor registers. None of this dust and debris was removed. On sites #1 and #8 urine was found in the ductwork. On sites #6 and #8 clogged toilets caused sewage to leak into air ducts during construction. Neither the urine nor sewage was removed.

Construction site personnel

Construction site personnel also contributed to potential problems. Greasy food residue deposited directly on wooden subflooring was noted to be common. In several instances soda had been poured onto the floor. On site #1 a half-full plastic soda bottle was intentionally nailed inside the wall prior to drywall installation.

Homeowners

Most homeowners neither regularly visited the construction sites, nor complained to the builder about any improper building practices. On all sites the exterior caulking had cracked during the first year of occupancy. None of the homeowners regularly checked their new houses for water leaks, allowing rainwater intrusion to go undetected for over extended periods of time. Crawlspaces remained damp because most homeowners did not regularly inspect them. Three homeowners let the interior temperature and humidity remain high in order to save on cooling costs.

DISCUSSION

The majority of the problems found were related to negligence and poor workmanship. These problems could have been avoided by better supervision, and by homeowners inspecting the construction sites regularly. However, some of the problems are more fundamental and require changes in design and construction practices.

Construction materials

Due to improper storage and handling many construction materials had visible fungal growth even before the materials were installed into the houses. The main causes were prolonged exposure to rainwater and direct contact with wet soil. The materials should be stored on raised beds to avoid contact with soil, and they should be properly covered. In humid climates where drying may be slow due to high relative humidity, it is important to control unnecessary moisture entry (Christian et al. 1991; Pasanen et al. 1992b). Exposure to food and beverage residues should also be avoided.

Heating, cooling, and ventilation

The problems with HVAC systems fall into two categories: inadequately sealed systems, and inadequately insulated systems. Leaving a system unsealed can enable contaminants and water to enter the system. Cooling coils and condensate drain pans of an unsealed HVAC system can quickly become an amplifier and a disseminator of fungal contaminants (Burge and Hoyer, 1990; ASHRAE, 1997; Pasanen et al. 1997). The checklist for sealed components includes plenum and distribution box corners and duct collars, air return duct collars, and openings for floor and ceiling air supply registers. The air filter should also rest on an airtight weather strip. If further reduction of circulated particulate contaminants is needed, both the air return and the air supply registers could have micro- particle filters installed. An inadequately sealed HVAC system can also be a major contributor to negative pressure within the building envelope.

Inadequately sealed bathroom, dryer, and range hood exhaust ducts allow exhaust air to escape inside ceilings and walls. The excessive humidity of this exhaust air may promote fungal growth on construction materials (Pasanen et al. 1992b). The ductwork must be inspected during the construction because eventually it will be encapsulated within the walls.

An inadequately insulated cooling system can lead to condensation problems on various structures around the house (ASHRAE, 1997). Constantly wet wooden surfaces can become significant sources for fungal contaminants within only a few months (Pasanen et al. 1991). To avoid condensation problems, every cold component of the system should have insulation and a vapor retarder, and metal components should never touch surfaces with even a moderate heat conductivity (ASHRAE, 1997). The check list for insulated components should include plenum and distribution box duct collars, and air supply registers and their boots. In high humidity areas plenums and air distribution boxes may require additional external insulation.

Negative pressure within the building envelope may cause crawlspace air to infiltrate the envelope. Infiltration can occur through unsealed floor joints, and openings on walls for plumbing and electrical outlets (Kelly, 1991). The construction materials inside the walls are exposed to the moisture and the contaminants (e.g., fungi, soil bacteria) originating from the crawlspace (Rautiala et al. 1996). The materials may then first become a reservoir, and later the amplifier for fungal contaminants. Negative pressure is created by HVAC systems, clothes dryers, exhaust fans, and central vacuum cleaners (Kelly, 1991). Negative pressure should be compensated by introducing replacement air in a more controlled way than infiltration. The building envelope should also be substantially airtight.

Drainage around the foundation

Improper drainage or grading around the foundation often leads to wet soil, or standing water in the crawl space (ASHRAE, 1997). Damp soil promotes fungal growth, sometimes to the extent that mushrooms can be found in the crawlspace. Evaporation of moisture can keep the air in the crawlspace humid even during low-humidity seasons. It is important to control moisture load in the crawlspace by proper drainage. Positive grading can be used to divert rain runoff away from the foundation (Moffatt, 1991). Problems with downspouts depositing large volumes of rainwater near the foundation perimeter can be corrected with extenders attached to the downspouts.

CONSTRUCTION DEBRIS

Wall structures should be clean of construction debris (e.g., sawdust and wood chips) when thermal insulation and drywall are installed. For example, sawdust can absorb large amounts of moisture and it provides a good growth media for fungi and other microorganisms.

Air ducts should be covered during construction. If air handling units must be operated during construction, special construction time filters should be used. If construction debris has entered the system, the debris should be removed before the system operates. Ductwork that has been exposed to urine, sewage or other liquids should be replaced.

HOMEOWNERS' RESPONSIBILITIES

Many of the construction time problems could be avoided if well-informed homeowners would visit the construction sites regularly. Complaining about improper practices usually causes the builder to take a corrective actions. After moving in, the homeowners should regularly monitor their homes for moisture and water leaks. The check list should include attic, crawlspace, garage, window frames, door frames, and visible cracks on exterior caulking. Homeowners should also monitor their homes for indoor air humidity levels, especially if they decide to save in cooling costs by not operating the air conditioning. In humid climates dehumidifiers may be useful in conjunction with the air conditioning in order to reduce excess household moisture (e.g., showering, cooking).

CONCLUSIONS

New houses clearly have potential fungal contamination problems, even before the construction work has been completed. Many of these problems could be avoided by educating builders as well as educating homeowners. Early identification and prevention of potential fungal reservoirs and amplification sites is preferable to costly analysis and repairs afterwards.

Moisture control is an essential component for healthy, durable and energy-efficient housing. Many significant improvements could be made by simple design changes and better workmanship. Building codes should include better enforcement measures for moisture control in buildings.

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A TOXIC MOLD CLEANUP GUIDE

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ABSTRACT

Many houses and small buildings are moldy, and some have extensive growth of toxigenic molds that can cause health problems at lower exposures than the phyloplane molds that are more common outdoors. Canada Mortgage and Housing Corporation, the Canadian federal housing agency, has performed a great deal of research into moldy houses and has created a number of publications on mold avoidance and cleanup. This document is one of a new series that addresses toxic mold cleanup as well as solving moisture problems and avoiding mold growth in the first place.

KEY WORDS

Mold, cleanup, houses, health effects, worker protection

INTRODUCTION

Canada Mortgage and Housing Corporation, the Canadian federal housing agency, has supported research into moldy houses, including the effect of mold on the health of children, for almost two decades. Health Canada's research (Dales et al. 1991,134) has shown that children are twice as likely to have reported respiratory disease symptoms when mold was reported. These findings are supported by over 20 studies from around the world, including a Canada/US study (Spengler et al. 1994). In the Canadian studies (Dales et al. 1991,134; Dales et al. 1991,143) there was also a cause and effect response, with more symptoms being reported when more mold was reported. In the field studies in Wallaceburg ON (CMHC 1994; CMHC 1995), that were performed by a team from CMHC, Health Canada and Dr. J. David Miller now with Carleton University, we were able to show a significant immune response in the school-aged children who lived in moldier houses. The effect was about the same size as passive smoking. Miller and Day (Miller, Day, 1997) summarize the evidence of the effect of mold on health quite clearly.

As a result of our work, CMHC has produced a number of publications on mold cleanup (NHA 6753, NHA 6789, CE7, CE8,) plus more on improved construction techniques (NHA 5031, NHA 5450, NHA 6915, NHA 6917, NHA 6919). More are underway, including the set of documents that includes this guide, *Toxic mold cleanup procedures: A guide for specialty contractors.* Two more are already in production, *Solving moisture problems in a home*, for renovators, and *Preventing moisture and mold problems in your home*, for householders.

Audiences for the guide

This guide is aimed primarily at the specialty contractor who will clean up serious mold contamination in a house or other small building, as well as the health officials who may order such a cleanup. Secondary audiences are: a) householders who wish to have such cleanups performed by others; b) renovators who either stumble into serious mold problems and wonder if they should proceed, or are solving moisture problems that led to a mold problem; and, c) building or health inspectors who find mold and wonder how to handle it. A tertiary audience is the building community and regulatory bodies who are still producing, or supporting the construction of, buildings that frequently get moldy.

Key assumptions

This document is based on a number of key: a) excessive exposure to mold can cause health problems, and exposure to some toxic molds can cause serious effects; b) the risk of health effects increases with exposure; c) the exposure increases with the total area of mold in the work area; and d) there are safe ways of dealing with even large mold problems, if sufficient protection is provided. The guide identifies three areas of mold at which a different level of protection is provided for the workers and occupants of the building. These are roughly based on The New York Protocol (NYC D of Health, 1993). It is further assumed that the thorough cleaning of the moldy surfaces is the primary method of removing the risk to mold exposure, and that disinfection of surfaces that remain in place must be followed by cleaning to remove dead mold material, since that material is still allergenic and toxic.

Establishing separation

One of the key ways of establishing protection for present and future occupants of a moldy building is to provide an effective separation between the work area and the remainder of the building. Such a separation requires: a) that a barrier is provided between the work area and the rest of the building; and, b) that the work area is depressurized relative to the area not affected. Because of the way buildings breathe, even a well-constructed a barrier does not provide enough protection in itself. This separation also provides worker protection as long as the worker does not transport contaminated materials or clothing away from the site when he/she exits the work area.

Worker safety

The primary steps used to provide worker safety involve respiratory and skin protection, along with protection for the eyes. In a large area cleanup the worker with be fully covered when on site and must be provided with a decontamination facility that will allow total separation of work and street clothing, following a thorough showering. Filtration of breathing air must be by HEPA filtration of respirable dusts with additional acid gas cartridges used when sodium hypochlorite is being employed for disinfection of moldy materials that are not removed from the building.

Cleanup procedures

The cleanup procedures require that moldy material that cannot be effectively cleaned must be bagged and removed from the site. This is particularly true of porous substances such as dry-

wall, carpets and upholstered furniture. For structural materials that cannot be economically removed and replaced, disinfection, cleaning and drying is required. Concrete cleaning is to be performed using tri-sodium phosphate (TSP) plus rinsing and drying. Sodium hypochlorite solutions are to be used for non-concrete surfaces when HEPA vacuuming is insufficient for cleaning to a point of effective disinfection.

Status

The guide is in the final production phases and is due for release early in 1999. Comments on the document are requested from those who use it, as a major rewrite is planned for later in 1999. It is only through in-field use that further improvements can be made, since the present version has already be out for three sets of wide-ranging reviews, and the majority of the comments received have been incorporated where that was possible.

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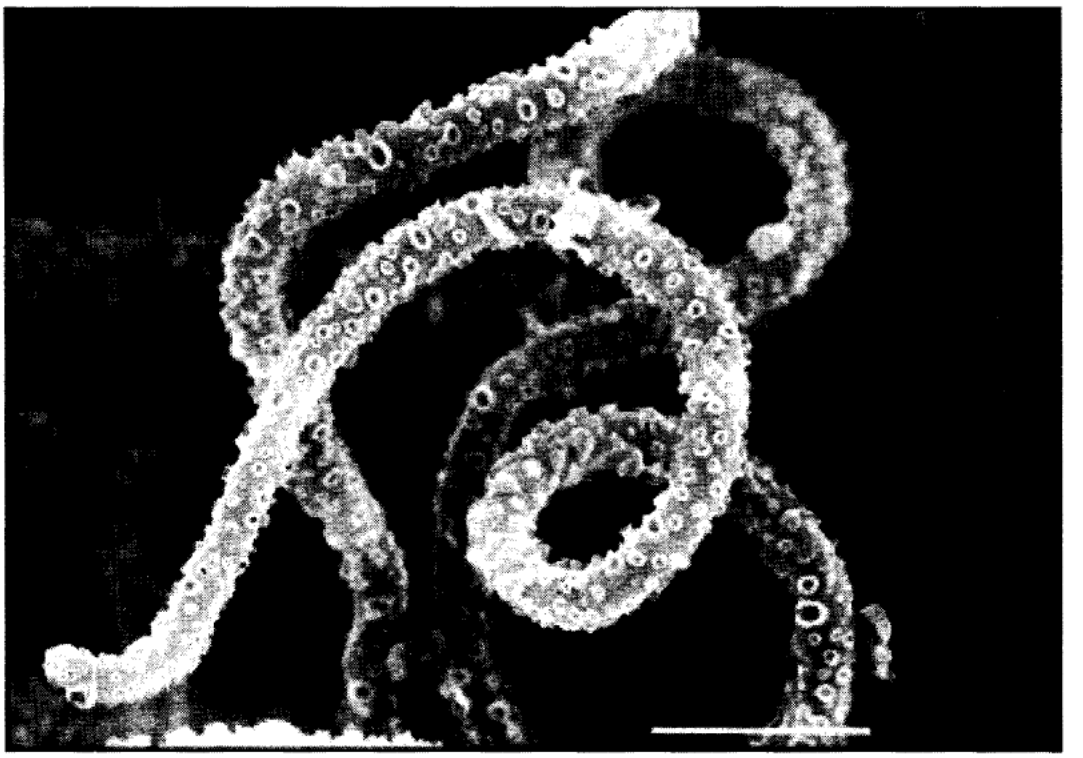
Stachybotrys atra, EM 3,300, ml 10 µm, dry wall, San Diego 1998, (E. JOHANNING, P.H. SIERCK); M. Gareis, Institute for Microbiology and Toxicology, Federal Center for Meat Research, D-95326 Kulmbach, Germany.



Chaetomium fumigatus, EM 2,300 x, ml 10 mm, gypsum board Albany, 1998, (E. JOHANNING, P.H. SIERCK); M. GAREIS, Institute for Microbiology and Toxicology, Federal Center for Meat Research, D-95326 Kulmbach, Germany.

Chapter 6

Public and Occupational Health Issues

DEVELOPMENT OF A PUBLIC HEALTH INTERVENTION PROGRAM IN FINLAND

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ABSTRACT

This paper presents the development and current stage of the national research program on moisture damaged and moldy buildings and related indoor air quality problems in Finland. The role of local, regional and national authorities as well as the current status of legislation and indoor air quality guidelines are described. Some needs for future research and the Environmental Health Research Program of the Academy of Finland are introduced.

KEY WORDS:

mold exposure, health effects, housing conditions, legislation, guidelines, research program, intervention study

INTRODUCTION

In Finland, moisture damages in homes, day care centers and schools became a question of public concern in the early 1990s. Before that, health risks associated with microbial exposure had mainly been studied in occupational environments, such as farming, sawmills etc. (Kotimaa et al. 1987, Kotimaa 1990). The sick building syndrome and the effects of the ventilation system on health had been studied in large office buildings (Jaakkola 1986, Jaakkola et al. 1991, Jaakkola et al. 1994). Several studies showed, however, that moisture problems and microbial exposure was a common problem in apartment buildings even in cold climactic conditions. Airborne concentrations of bacteria and mold were measured in different types of buildings during different seasons of the year (Nevalainen 1989, Reponen et al. 1992ab, Pasanen et al. 1992).The first major complaints of moisture damage and mold growth came from rental apartments in community owned blocks of flats and detached houses in large cities. In these apartments, a number of inhabitants experienced respiratory symptoms and infections associated with living in apartments with visible mold growth. A questionnaire was sent to all Finnish municipalities asking whether the authorities of environmental health had received complaints concerning moisture damages and/or mold growth in apartment buildings. Over 70 municipalities were aware of several mold damaged buildings in their area, and these included both public buildings, work places and rented and privately owned apartment buildings and one family houses. Most of these reports came from southern Finland where a vast majority of the population is living. Local and regional authorities in municipalities, cities and counties faced a

wide-spread problem of microbial exposure with sparse resources and knowledge on the subject.

In 1990, the first pilot studies on health effects associated with microbial exposure in apartment buildings and day care centers were started in the Division of Environmental Health of the National Public Health Institute (KTL). These pilot studies showed high prevalences of respiratory symptoms and disorders among children and adults (Husman et al. 1993, Koskinen et al. 1995, Koskinen et al. 1997). Thus, a national research programme was started in the KTL involving the Laboratory of Environmental Microbiology, Unit of Epidemiology and later also the Department of Toxicology. The KTL research program is nationwide and has close collaboration with Regional Institutes of Occupational Health in Kuopio and Tampere, Department of Environmental Sciences at Kuopio University, Kuopio University Hospital and the State Technical Research Center. Experts on construction engineering have joined the multidisciplinary working group which now consists of engineers, microbiologists, hygienists, epidemiologists, sociologists, toxicologists, pediatricians and oto-rhino-laryngologists.

KTL is a state research institute subordinated by the Ministry of Social Affairs and Health. The "Moldy Houses" research program was originally funded by the Ministry of Social Affairs and Health and the Ministry of Environment. In recent years, also the Finnish Work Environment Fund and the Academy of Finland have funded the program. Also the parallel research programs on construction engineering and technology and HVAC-systems at the Technical Universities in Helsinki and Tampere have completed the multidisciplinary approach to solve the problem of moisture damaged buildings.

THE AIMS OF THE MOLDY HOUSES RESEARCH PROGRAM

The aims of the research programme has been to reveal the prevalence and causes of water damages and moisture problems in Finnish housing stock, to describe the quantitative and qualitative characteristics of microbial exposure in apartment buildings, day care centers and schools and identify the adverse health effects associated with microbial exposure in different populations. In recent years, the aims have been to characterize the mechanisms of moisture damages and the growing conditions of moisture related microbes in buildings (KTL and the University of Kuopio). In a number of studies on health effects, epidemiology and pathophysiology of microbe related disorders and diseases are studied in populations, individual patients as well as among workers in school buildings, day care centers and construction workers exposed in remediation of moldy buildings (KTL, University of Kuopio). These include also studies conducted at the clinics of dermatology and lung diseases at the Kuopio University Hospital. The mechanisms of microbe related disorders, especially inflammatory processes and allergy will be further studied on cell cultures and animal models in experimental exposure as well as on patients in different types of work exposure. The ongoing studies include also characterization of allergens, especially *Stachybotrys chartarum* (University of Kuopio), the significance of microbe specific IgG- and IgE-antibodies (Kuopio Regional Institute of Occupational Health), case control studies of asthmatic children with special reference to mold exposure (KTL), and the use of PCR-techniques in characterization of microbial growth in the buildings (KTL). One aim is also to reveal the social and psychological mechanisms of deci-

sion making in communities handling moldy buildings (University of Kuopio) and to estimate the socioeconomic costs and consequences of the mold problem in Finland (National Agency for Welfare and Health) (Nguyen et al. 1998).

A major part of the program was focused on intervention studies, which follow the conditions in the building, the quality of indoor air and the health status of an exposed population before, during and after the remediation of moisture damages (Technical Universities, KTL). Ongoing studies include schools, day care centers, university buildings and hospitals (Haverinen et al.).

SUMMARY OF THE RESULTS

The pilot studies revealed a high number and a wide variety of moisture damages and associated microbial growth in apartment buildings and day care centers (Hyvärinen et al. 1993). The research program was continued with a random sample of single houses and terraced houses in different parts of the country in order to identify the prevalence of moisture damage and the typical mechanisms of moisture damage in buildings from different decades. A similar study was carried out in a random sample of apartment buildings. The results were similar in independent studies, approximately three quarters of the buildings had had some type of moisture damage during the years. The cumulative incidence was similar in apartment buildings, day care centers and school buildings (Nevalainen et al. 1998, Ruotsalainen et al. 1995, Kurnitski et al. 1996).

Adverse health effects were clearly and consistently shown in exposed populations in children and adults, in apartment buildings, schools, day care centers and other work places including university buildings and remediation work (Husman et al. 1993, Koskinen et al. 1995, Koskinen et al. 1997, Pirhonen et al. 1996, Taskinen et al. 1997, Rautiala et al. 1998). Preliminary results from the intervention studies show a considerable decrease in respiratory symptoms and infections as well as use of antibiotic treatment and health care facilities after the remediation of a moisture damaged school building (Haverinen et al. 1997, Savilahti et al. submitted). Preliminary results also show an association between mold exposure and mold allergy and asthma in children (Taskinen et al. 1997). In experimental studies, cytotoxicity and induced production of nitrogen oxide and cytokines has been found due to exposure with *Stachybotrys* sp. and actinomycetes (Hirvonen et al. 1997, Ruotsalainen et al. 1995).

TRAINING PROGRAMS

In coordination with the research programs, a training program with courses for health inspectors and construction engineers and physicians was conducted in cooperation with FIOH, the University of Kuopio as well as with Technical Universities and Ministry of Social Affairs and Health. Local authorities of environmental health surveillance in municipalities are health inspectors and hygienists. Health inspectors have been trained on courses given by the University of Kuopio and KTL in sampling, cultivating and identification of microorganisms typically growing in moisture damaged buildings. Nowadays, several laboratories in larger cities are able to culture and identify at least the most typical microorganisms in moisture damaged buildings. These laboratories consult the microbiological laboratories of the KTL and the

Kuopio Regional Institute of Occupational Health. Construction engineers have been trained in Technical Universities and Institutions to be experts in building inspection and moisture damages. In the Finnish Institute of Occupational Health, occupational health physicians and nurses have been trained to identify symptoms and signs of indoor air problems in work places. Physicians working in outpatient care and hospitals have been trained in courses given by Kuopio University, KTL and FIOH. General principles of clinical examination and treatment of mold exposed patients have been published in the Finnish Medical Journal (Nordman and Mussalo-Rauhamaa 1998).

LEGISLATION AND GUIDELINES

Along with the research and training, new laws and guidelines have been prepared. According to the Finnish legislation, the local authorities responsible for risk assessment are health inspectors with training of a hygienist. The leading officer of health surveillance and environmental health is a veterinarian in most municipalities and cities in Finland. The first indoor air guideline came out in 1990 based on the previous Health Care Act (Murtomaa and Aurola 1990). This guideline stated for the first time that visible mold growth in residences is a health hazard. The principles of action in municipal level in indoor air problems are based on indoor air guidelines given out by the Ministry of Social Affairs and Health.

The Health Protection Act (former Health Care Act) was renewed in the year 1995 and the national guidelines on indoor air quality and on measurement of indoor air exposures was rewritten and published in 1997 (Health Protection Act 1994, Indoor Air Guidelines 1997, Aurola and Välikylä 1997). In residences and related buildings, the condition of the building and possible health risk associated with exposures are the responsibility of the owner, but the environmental health authorities in municipalities may require remediative actions to remove or minimize health risks. In extreme situations, it is possible to prohibit the use of the building because of a health hazard. A health hazard is defined in the Health Protection Act as "a sickness or another disturbance of health in a person or the occurrence of a factor or condition that may reduce the healthiness of the living environment of an individual or population"(Section 1, Health Protection Act). The health requirements for dwellings and moisture damage and microbial exposure is defined in the Section 26 of the Act: The purity of the indoor air of a dwelling and other indoor premises, their temperature, humidity, noise, ventilation, light, radiation and other corresponding conditions shall be such that they do not constitute a health hazard to those staying in the dwelling or the indoor premises. A dwelling and other premises used by people shall not contain animals or microbes to an extent that they would constitute a health hazard (Health Protection Act 1994).

THE USE OF QUESTIONNAIRES AND THE DIAGNOSIS OF DISEASES ASSOCIATED WITH MOLD EXPOSURE

Suspected health effects can be examined by questionnaires delivered to the users of the building, both workers and other people, children, patients or clients depending on the situation. The s.c. MM-40 questionnaire has widely been used in different indoor air problems. Also

other questionnaires are available for adults and children. In Finland, a national Tuohilampi-questionnaire is available for adults (Susitaival and Husman 1996). These questionnaires have been used not only in research projects but also in primary health care as a screening tool in solving indoor air problems.

The expert group of KTL gives advice to local authorities in the whole country. The five regional Institutes of Occupational Health (Uusimaa, Turku, Lappeenranta, Tampere, Kuopio and Oulu) help occupational health physicians in each part of the country. Kuopio Regional Institute of Occupational Health serves the whole country in analyzing the microbe specific IgG- and IgE-antibodies in serum samples. The analysis of microbe specific IgG-antibodies as a measure of exposure is available for 42 moisture related microbes. Over 12 000 serum samples have been analyzed during the years 1980-1997 (Reiman et al. 1998).

The clinical diagnosis of occupational diseases of mold exposed patients takes place mainly in the Finnish Institute of Occupational Health in Helsinki. According to the National Statistics of Occupational Diseases, approximately 500 new cases of occupational diseases due to mold exposure have been diagnosed annually during recent years. These diseases include mainly allergic rhinitis, asthma, alveolitis and organic dust toxic syndrome associated with microbial exposure in moisture damaged buildings (Karjalainen et al. 1997). Patients exposed in non-occupational settings are examined in two clinics specialized for indoor air exposures, these are the Indoor Air Clinic at the Skin and Allergy Hospital Helsinki and the Dermatology Clinic of Kuopio University Hospital.

INFORMATION CAMPAIGNS

The aim of information campaigns has been the early detection and prevention of moisture damage and related mold problems, improved maintenance of the buildings as well as immediate and proper remediative action in damaged buildings. Information campaigns and annual seminars coordinated by the Finnish Society of Indoor Air Quality and Climate (FiSIAQ) have been invaluable in distributing information among professionals and the public.

FUTURE ACTIVITIES

The Academy of Finland has started the Finnish Research Programme on Environmental Health (SYTTY) in August 1998. A total of 11 million out of 47 million FIM will be used in indoor air research. The forthcoming research projects will continue intervention programs in school buildings, reveal the health consequences as well as pathophysiologic and allergologic mechanisms behind the mold exposure in experimental and clinical materials. Multidisciplinary approach will include experts in health economy, sociology and public decision making in the program (Juuti and Leinonen 1998).

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AN ASSESSMENT OF MOLD CONTAMINATION PROBLEMS IN ATLANTIC CANADA SCHOOLS: MOLD BURDENS, AMPLIFYING SITES AND BENEFITS OF PROACTIVE SCHOOL INSPECTION POLICIES.

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ABSTRACT

It is abundantly clear that many schools support unacceptable mold burdens. However, what is less clear from the literature on school biocontamination problems is whether all schools support the same types of molds and where the most common mold amplifying sites are likely found. Since 1992, I have been involved in the study of 631-school environments for mold contaminants and analyzed some 5000 air and substrate samples from schools throughout the Atlantic Provinces. Objectives of this presentation are threefold. The first is to compare mold burdens and dominant species assemblages in "normal" and "contaminated" school environments. The second is to show that mold species assemblages vary widely with the school construction type and amplifying sites and; the third is to evaluate the effect of ongoing inspections in schools for mold contaminants. In normal school environments, like in other buildings in temperate zones, airborne mold burdens follow clear seasonality patterns that are quantitatively and qualitatively similar to that found outdoors. Schools suffering from mold contamination problems most frequently support high focal and sometimes widespread spore loads and growth of a variety of species including the indicator species *Acremonium spp., Aspergillus fumigatus, A. niger, A. ustus, A. versicolor, Chaetomium spp., Paecilomyces variotii. Penicillium brevicompactum, P. aurantiogriseum complex, P. variabile, Phoma spp., Stachybotrys chartarum, Trichoderma harzianum* and *T. viride*. However, indicator species assemblages recovered from contaminated school sites vary considerably reflecting school age and construction, and the composition and water activity of the amplifying substrate material. The most common contamination sites in descending order of occurrence are classrooms and administration sites, especially peripheral-wall cavities and ceiling plenum, libraries, gymnasiums, basements/crawl spaces, ventilation systems, janitor/mechanical rooms and portable classrooms. The most commonly encountered contaminated substrates in schools are drywall, dust, ceiling tiles, structural wood, insulation, carpets, concrete wall surfaces, and, amongst others, that have been exposed to chronic water infiltration, condensation and/or wicking problems. School inspections for mold contamination, which have been ongoing since 1993, have resulted in a reduction in the number of schools suffering from widespread contamination problems. However, the number of schools suffering from focal problems, especially in inapparent sites, has remained approximately the same. Prompt abatement action to remove amplifiers once they have been detected, and better communication outlining potential exposure risks, have helped to allay heightened occupant and parent anxiety.

INTRODUCTION

It is abundantly clear that building environments can suffer from mold and bacterial contamination problems. It is also clear that these contaminants impact on building air quality, and the comfort and health of the occupants.

Since 1992, I have been conducting mycological investigations of Atlantic Canada schools, or have been analyzing samples collected from them for mold species. As a general comment, I can safely say that microbial contamination of schools in this region is a real and significant problem. Building contamination by molds has affected air quality, resulting in both permanent and temporary school building closures, and has been linked to health problems especially among some children and teachers, details of which we have presented in one of our posters (Wiseman and Rand, 1998).

Since 1993, I have argued that one way by which mold-related air quality issues and comfort and health problems in schools could be allayed is to have informed school board representatives and/or Department of Health officials mount their own microbial investigations (Rand, 1994). These would be part of their on-going IAQ assessments of school properties. Colleagues and I have taken this message to many meetings, with some success. However, several things still stand clear. Many school board representatives and Health officials still remain uncertain as to what constitutes mold contamination, where contamination is likely to be found in schools, and whether their actions in mounting their own investigations have had any effect, especially in reducing complaints within school properties for which they are responsible.

The objectives of this presentation are threefold. The first is to provide summary information on mold burdens and assemblages in normal and problem building environments. The second is to provide an overview on mold burdens in different types of schools and identify the various potential amplifying sites within these building types. The last is to evaluate the success of the proactive inspection policies within Atlantic Canada schools.

MATERIALS AND METHODS

Some 4886 samples from 631 schools from across Atlantic Canada were used in this study. Schools were either sampled as part of routine IAQ investigations (ambient sampling), or were sampled as a consequence of contamination (complaint-based) concerns. Sampling was done either under normal conditions, when sites were occupied or after hours during the evening when the school was closed, and in accordance with AIHA sampling protocol (Dillon et al. 1996).

Sampling for airborne molds from all schools was performed using a RCS Biotest sampler using RoseBengal medium sampling strips to determine CFU/M3 and species composition. Sampling time was for 4 or 2 minutes depending on sample site. Sampling for substrate samples involved the removal of materials from apparently contaminated sites and crumbling approximately 1-cm^2 or 1-cm^3 pieces or about 0.1-g dust directly onto 2% MEA medium for species enumeration and identification. All samples were incubated at 25°C for 3 -7 days for enumeration. For identifications, fungal isolates were either identified directly from the RCS or MEA plates. *Aspergillus* and *Penicillium spp.* isolates were subinoculated onto CYA and MEA

(and CREA and YES, as needed (see Samson et al. 1996) and incubated at 25°C for an additional 7 days for species identifications.

For analyses, sample year and month were both recorded for all samples. Schools from each Province (1 = New Brunswick; 2 = Newfoundland; 3 = Nova Scotia; 4 = Prince Edward Island) were segregated into four construction types as follows: (1 = wood frame; 2 = masonry; 3= steel frame; and 4 = masonry/steel frame buildings), where knowledge allowed. All school sampling sites were stratified from 1 to 11 as follows: (0 = outside air; 1 = classroom; 2 = administration; 3 = crawl space/basement; 4 = ceiling plenum; 5 = wall cavity; 6 washroom; 7 = ventilation system; 8 = library; 9 = gym; 10 = janitors office/mechanical room; 11 = portable classroom). Additionally within each of these sites different sampling substrates were identified as follows: (0 = air; 1 = ceiling tile; 2 = drywall; 3 = insulation; 4 = soil; 5 = cement wall; 6 = structural wood (wood panel/joist/stud); 7 = carpet; 8 = dust accumulations). Lastly, sites supporting individuals suffering complaints consistent with mold exposures was also noted from questionnaire responses and self-reported symptoms (0 = no complaints; 1 = complaint). These factors were then be correlated with viable spore loads of the 47 of the most frequently occurring species isolated from the schools, including phylloplane, soil dwelling and the notoriously toxigenic species. Data were analyzed using Systat 7.0 and, where appropriate, were considered significant at the $P < 0.05$ confidence level.

RESULTS AND DISCUSSION

Table I shows that the four types of schools from the four Atlantic Provinces were well represented in the sampling. Highest numbers of school sites sampled were from Nova Scotia while fewest were from Prince Edward Island. The highest number of school samples were from masonry buildings while the lowest number represented samples taken from wood frame and combination construction type buildings.

TABLE I: Sample sizes from four different school types in Atlantic Canada provinces.

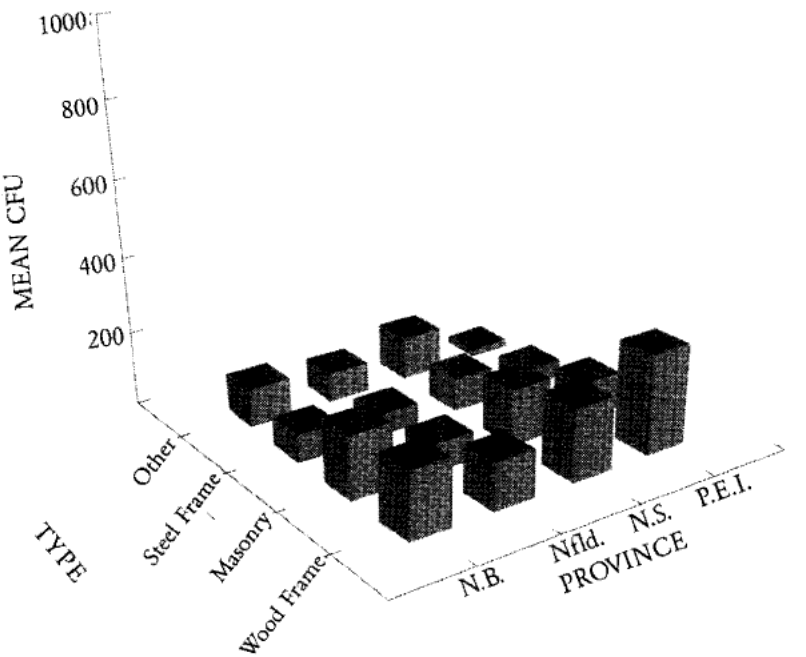
	PROVINCE				
	N.B.	NFLD.	N.S.	P.E.I	TOTAL
SCHOOL TYPE					
Wood Frame	117	264	266	63	710
Masonry	579	687	1040	381	2687
Steel Frame	72	238	345	115	770
Other	198	70	384	67	719
TOTAL	966	1259	2035	626	4889

Numbers are sample sizes for each school type and from each Atlantic Canada province

Comparison of ambient air samples taken from these building types reveals that spore burdens in wood frame and masonry types were significantly higher ($P< 0.000$) than in steel frame and combination school construction types (Figure I). Spore loads in wood frame and masonry schools may be elevated because they are generally older and have suffered more from build-

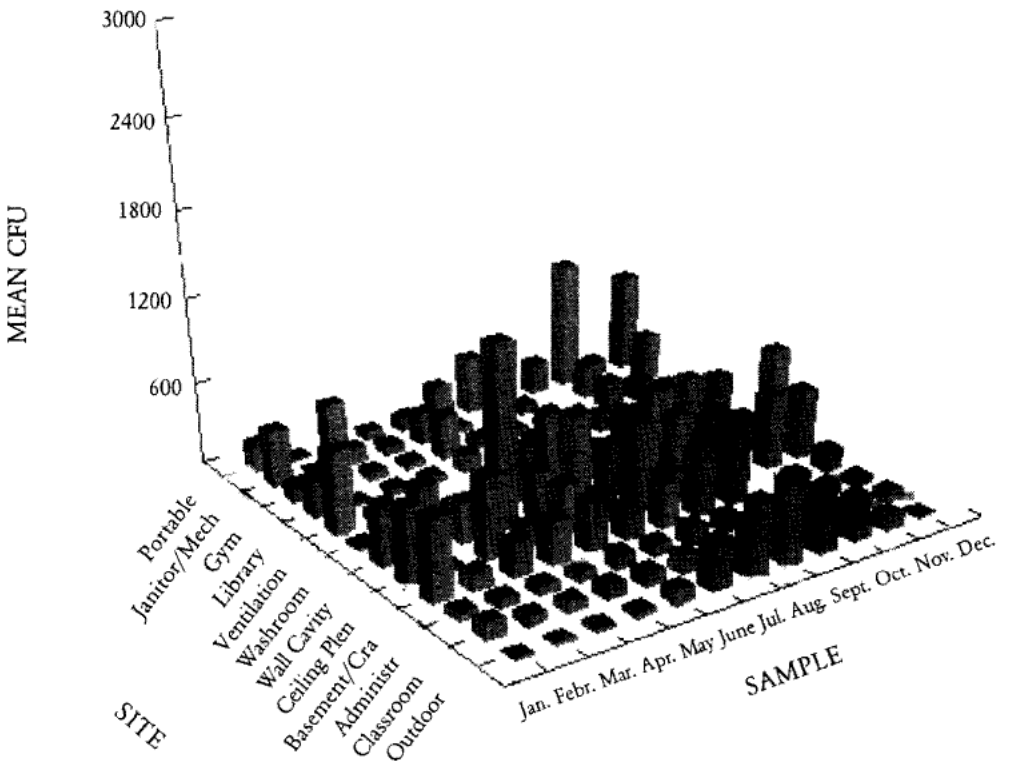
ing envelope (roof and wall) deficiencies and building material deterioration due to water infiltration and condensation problems than the other school types. Moreover, wood frame and masonry type schools often support higher occupant densities and suffer from storage problems, which can lead to airflow occlusion and condensation problems leading to mold amplification along peripheral walls. These schools also often suffer from cleaning problems that lead to litter and dust accumulation, which in turn can serve as mold reservoirs and potential amplifiers.

FIGURE I:. Mean CFU/m³ spore loads in four different school types from Atlantic Canada provinces.



Evaluation of mean spore loads recovered from different school occupancy and non-occupancy sites reveals several trends (Figure II). The first is that spore loads in occupancy sites follow seasonal trends, which reflect the outdoor seasonality profile. Lowest mean indoor spore loads were encountered in late fall through to early spring months. Highest mean spore loads were from spring through to fall months. Indoor spore loads in schools in fall through to early spring were higher than the outdoor loads, and lower from spring to fall months compared to outside levels. These results are consistent with those of earlier studies involving buildings in temperate regions (see Li and Kendrick, 1995). They reinforce the position that spore burdens in building environments should be equal to or less than outdoor spore loads from spring through to fall months and higher than outdoor loads in fall through to early spring.

FIGURE II: Seasonal changes in mean CFU spore loads from outdoor air and different collection (occupancy and non-occupancy) sites in schools in Atlantic Canada.



Spore loads in non-occupancy spaces were higher than those in occupancy spaces, and did not show distinct seasonal variation. Highest spore loads in non-occupancy spaces were in ceiling plenums, basements/crawl spaces and peripheral wall cavities. This difference between mean spore loads in occupancy and non-occupancy spaces is probably because spores in occupancy spaces are transitory and entrained from outdoor sources, human traffic, insects, building material surfaces, dust and non-occupancy spaces. In non-occupancy spaces, spore loads are probably due to the accumulation over time of spores on/in building materials due to their impaction and settlement on non-occupancy space materials. In my experience, as non-occupancy spaces can accumulate high spore loads they also constitute primary fungal-spore reservoirs in school building environments. Also notable is that washrooms are often sites supporting high mean spore loads. This may because they are often subject to plumbing leaks and condensation problems, and wicking along the base of their wall surfaces due to careless wet mopping by custodial staff, amongst others, which serve to support mold growth.

It is important to emphasize here the point made earlier at this meeting by Dr. Miller (Pers. comm. 1998) that reports on CFU levels in buildings are unimportant, at least in the context of evaluating whether a building is normal or suffering from bioamplification/ contamination

problems. Far more important and useful is an evaluation of the species assemblage associated with building samples.

In general, the mold species composition in normal schools and ones suffering water condensation/infiltration problems varies greatly. If we accept the theoretical position that normal school environments are ones that do not support toxigenic fungi, the proportion of soil-dwelling species (*Aspergillus* spp., *Penicillium* spp., etc.) to phylloplane ones (*Alternaria* spp., *Aureobasidium* sp., *Cladosporium* spp., non-sporulating isolates, *Ulocladium* spp., yeasts, etc.) is low. Phylloplane species numbers also exceed soil-dwelling species numbers (Figure III). This figure closely resembles that provided by Miller (1992) and Flannigan and Miller (1994), which shows the proportion of soil-dwelling to phylloplane species in audit samples from Canadian buildings. Moreover, the prevalence of species in normal indoor samples is qualitatively similar to that encountered in outdoor samples (Table II). These results support the position that in normal schools, the mold species assemblage should be similar to that encountered outdoors.

FIGURE III: Proportionate relationship between filamentous mold species (primarily *Aspergillus* and *Penicillium* spp.) and phylloplane species from normal school collection sites. Numbers along x and y-axes are spore loads expressed as CFU/m³.

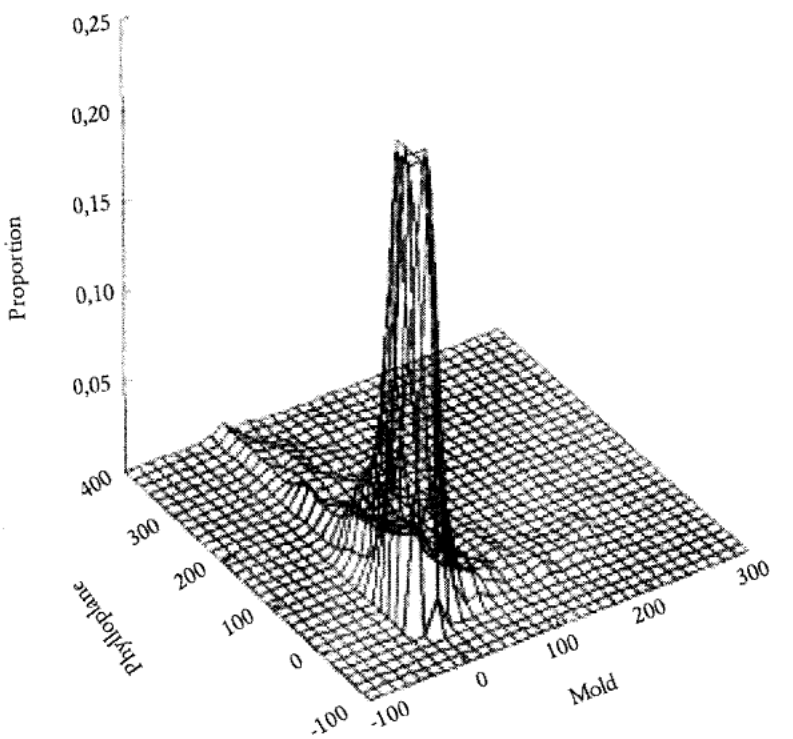


TABLE II. Frequency of occurrence (%) of commonly encountered mold species in outdoor and healthy indoor Atlantic Canada School air samples.

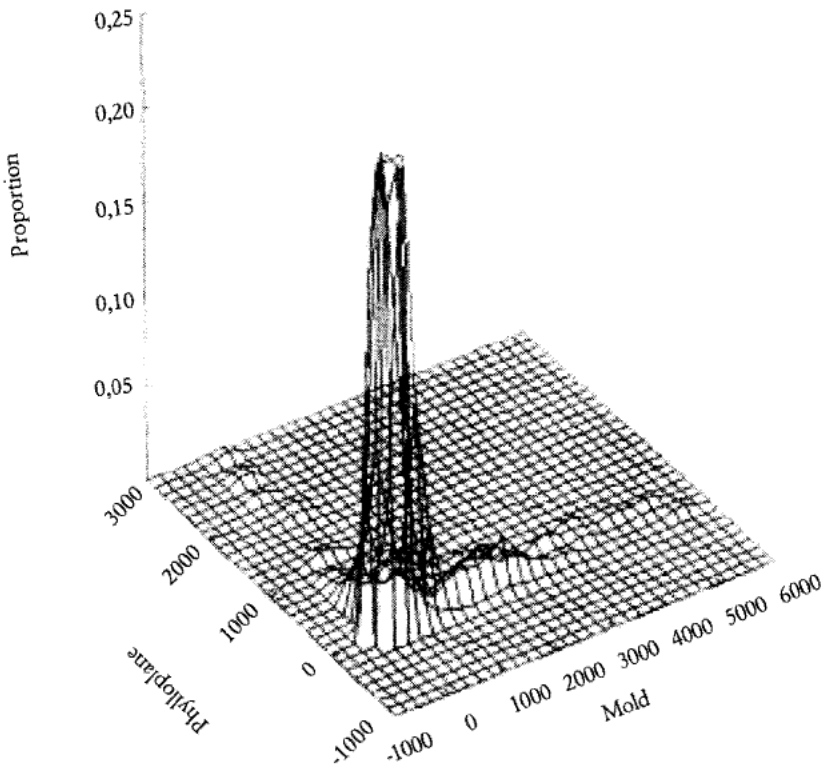
	OUTDOOR	INDOOR
Alternaria alternata	27.0	23.7
Aureobasidium pullulans	16.8	15.4
Clad. cladosporioides	50.9	57.1
C. herbarum	34.1	32.3
C. sphaerospermum	15.6	17.4
Epicoccum nigrum	11.3	12.1
Penicillium chrysogenum	39.9	35.4
P. commune	8.8	17.2
P. expansum	11.3	7.3
N.S.I	34.7	31.6
Ulocladium chartarum	27.6	23.6
Yeasts	30.1	31.4

In schools that support at least one viable spore of a toxigenic species (*Aspergillus fumigatus, A. versicolor, Paecilomyces variotii, Penicillium aurantiogriseum, P. brevicompactum, P. viridicatum, Stachybotrys chartarum, Trichoderma harzianum*, and *T. viride*) and which have been suffering from moisture problems, the proportion of soil-dwelling species/phylloplane species is high. They also often support a species assemblage comprising predominately soil dwelling species, including the above-listed toxigenic mold taxa (Figure IV). In these schools, soil-dwelling species spore loads exceed phylloplane species spore loads, which are also elevated compared to normal building substrata. Additionally, the prevalence of species associated with indoor sites suffering wetness problems is dissimilar to that encountered in outdoor samples (Table III). These results support the position that in non-normal schools, the mold species assemblage is quantitatively and qualitatively dissimilar to that encountered outdoors, and in normal school environments.

TABLE III: Frequency of occurrence (%) of some indicator mold species in outdoor and unhealthy-indoor Atlantic Canada School air samples.

	OUTDOOR	INDOOR
Acremonium strictum	2.0	10.8
Aspergillus fumigatus	2.1	5.6
A. niger	1.3	11.9
A. ustus	0.05	10.7
A. versicolor	5.6	34.6
Chaetomium aureum	0.03	9.3
Paecilomyces variotii	0.01	12.6
Penicillium aurantiogriseum	1.5	20.7
P. brevicompactum	1.9	11.9
P. viridicatum	0.01	15.1
Pithomyces chartarum	0.3	11.3
Stachybotrys chartarum	0.00	22.6
Trichoderma harzianum	0.01	12.8

FIGURE IV. Proportionate relationship between filamentous mold species (primarily *Aspergillus* and *Penicillium* spp.) and phylloplane species from school collection sites suffering from water infiltration/condensation problems. Numbers along x and y-axes are spore loads expressed as CFU/m³.

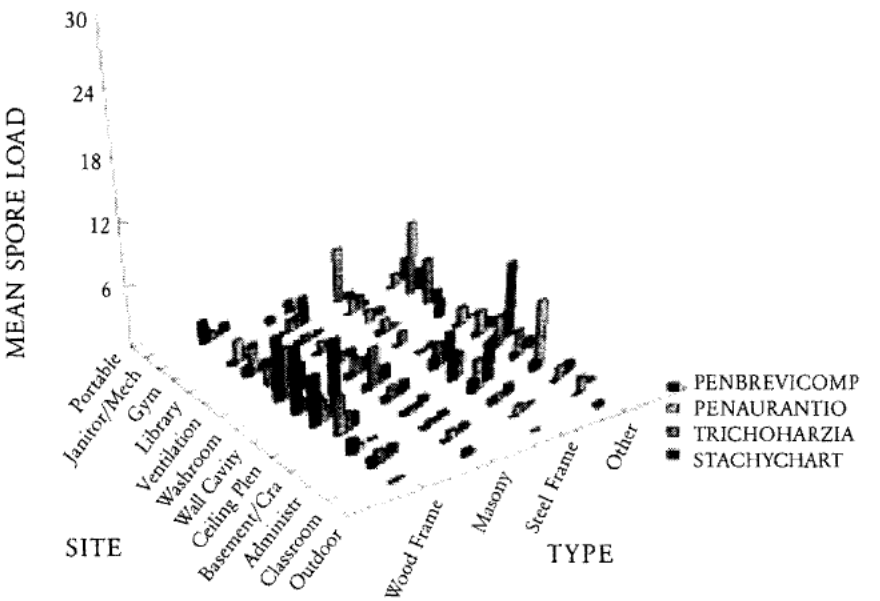


Without first-hand, on-site-inspection-based information, it is often difficult to determine soil-dwelling species amplification sites within school environments. Nevertheless, building survey-sampling results can provide some useful clues to probable areas. For example, comparison of prevalence and spore loads of *S. chartarum* reveals that while spores of this species are likely

prevalence and spore loads of *S. chartarum* reveals that while spores of this species are likely
found in any school suffering from chronic wetness problems, including ambient air, highest spore loads are in peripheral wall cavity and ceiling plenum sites (Figure V). Additionally, highest *S. chartarum* prevalence and mean spore loads are associated with ceiling tiles and drywall suggesting if these materials support high water activity (aw > 0.90) they may serve as primary amplifiers. On the other hand, *Trichoderma harzianum*, which is a wet-loving species like *S. chartarum*, is generally found on structural wood elements (e.g. wall joists, windowsills, doorjambs) suffering water deterioration problems. Furthermore, *Penicillium aurantiogriseum* and *P. brevicompactum*, which are both mesophilic species and relatively common in building suffering mold-related biocontamination problems, generally occur on different building substrates. *Penicillium aurantiogriseum* is frequently recovered from drywall and concrete surfaces while *P. brevicompactum* is commonly recovered from structural wood elements suffering from water deterioration. This

result indicates that these last two-mentioned mold species differ with respect to their nutritional requirements and substrate preferences. Therefore, in my experience, the recovery of toxigenic species and subsequent identification of amplification sites in school buildings suffering from moisture problems can often be achieved with an understanding of building design and history, and a knowledge of the nutritional and water activity requirements of the isolated fungus.

FIGURE V: Mean spore loads and prevalence of 4 toxigenic mold species in outdoor air and from different Atlantic Canada school building sites.

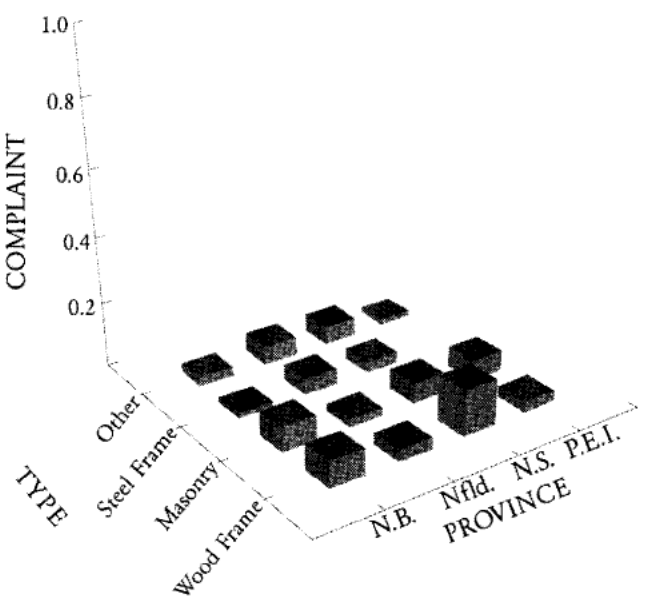


In this conference, we have heard many times that exposure to molds can lead to a variety of health complaints and problems. In Atlantic Canada, school contamination by molds has been the focus of much attention by the media, school boards, school occupants, parent groups and the medical profession. However, what is clear is that tolerance for contamination in schools still varies considerably amongst these interested parties. In my experience, school occupants, School Occupational Health and Safety Committees and parent groups show little, if any, tolerance toward the presence of toxigenic molds in their schools. On the other hand, certain members of the medical profession still view mold contamination issues and the potential impact that molds may have on school occupants skeptically. Indeed, The Advisory Committee on Environmental Hypersensitivity to the Nova Scotia Department of Health recently (1997) concluded "... statements implicating molds as causing significant illness in Nova Scotia school children are superficial, leaving unstated the conditions and circumstances of the reported fungal growth, and are at variance with current knowledge." In my opinion, this position reveals a

certain lack of first-hand knowledge and understanding of the conditions in some schools suffering from biocontamination problems.

During the course of our investigations in schools for mold contamination, and on the basis of questionnaire responses, self-reported symptoms and medical diagnoses, we have found that some school occupants have suffered health problems consistent with exposure to molds. These problems include upper respiratory problems, runny noses, headaches, rashes, nosebleeds and reaction to musty odors. Additionally, my data reveal a positive correlation between complaints and the presence of certain toxigenic molds in schools. The strengths of association range between 0.201 for *Penicillium aurantiogriseum* and 0.508 for *Trichoderma harzianum*. Highest complaint prevalence is associated with wood and masonry frame buildings (Figure VI), which are the same school types suffering from age-related building envelope deficiencies, crowding, storage problems, amongst others. Additionally, complaints are voiced throughout the year, although highest complaint prevalence is in fall and winter months, coinciding with when students are occupying schools that have their windows closed due to the cold or inclement weather.

FIGURE VI: Complaint prevalence in four different Atlantic Canada school types.

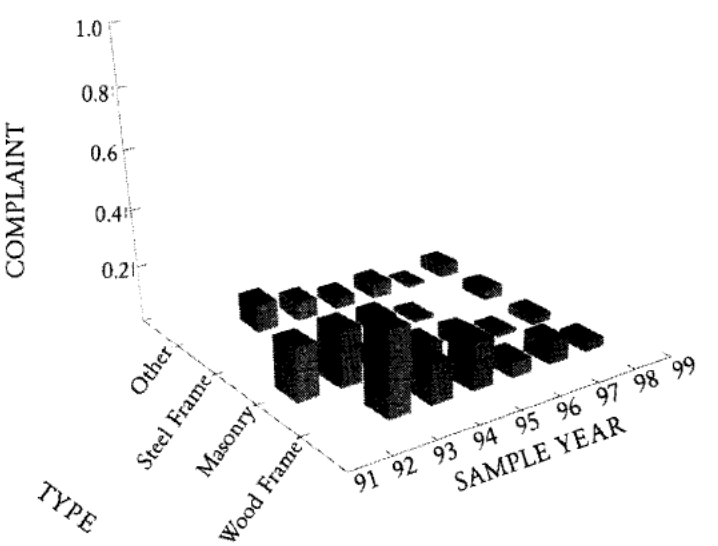


From 1992 to 1993, the prevalence of comfort and health complaints in schools throughout Atlantic Canada was relatively high. Initially, the high number of complaints resulted in reactive sampling approaches to determine whether the school was suffering from mold contamination problems. Because they often were, this led to a growing awareness that something must be done to address the underlying problems. This was probably motivated by the need to reduce the high costs associated with reactive-based, mold-related school inspection and abatement actions, and to allay student-teacher-parent concerns and anxieties. It was also motivated in

order to satisfy Federal and Provincial Health and Safety regulations (see Anon 1994) to comply with worker and building occupant safety issues. In 1993-1994, the reactive approach shifted both philosophically and practically to a proactive one.

The proactive approach offered the recognition that mold contamination in schools is unacceptable from both building performance and occupancy health and comfort perspectives. It stimulated school boards and school maintenance workers to keep their properties clean and free of mold contamination. It also stimulated increased worker IAQ training, and has resulted in routine sampling and building inspections for fungi. It has also resulted in School Board or the Department of Health (Prince Edward Island) workers responding promptly to complaints by investigating possible causes so that appropriate corrective actions can be taken, based on recommended abatement strategies. However, a question is whether this proactive approach is working. While it is difficult to determine how to evaluate the success of such actions by the school boards, one method is to compare the prevalence of mold-complaint based sampling in schools throughout the region. The result of this has been a significant decline in the number of complaints consistent with mold exposure in schools from over 20% to less than 10% (Figure VII). This result reveals that the actions taken have had marked success. However, as complaints and mold problems persist, it is important to maintain strict vigilance to achieve further environmental quality improvements in Atlantic Canada schools.

FIGURE VII: Change in complaint prevalence in Atlantic Canada schools from 1992 to1998.



CONCLUSIONS

- Schools supporting highest mold spore burdens are most likely wood frame and masonry types, reflecting school age, condition and occupant densities.
- Normal school environments support predominately phylloplane species identical to the ones outside. Dominance of indoor samples of non-phylloplane species, especially toxigenic ones, indicates that the building is likely suffering from water infiltration/ condensation problems.
- The nature and location of mold contaminated substrates within schools can usually be determined by an understanding of building design and history, and the water activities and nutritional requirements of the isolated fungi.
- Occupant complaint prevalence associated with molds in schools is highest in wood and masonry frame buildings, and in the spring and fall months.
- School occupant complaints associated with mold exposures have declined markedly from 1992 to the present, indicating the usefulness of the proactive inspection policies adopted by Atlantic Canada School boards.

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Special Section.

MOLDS AS AN ENVIRONMENTAL FACTOR IN INFANT LEUKEMIA?

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OBJECTIVE

The possible influence of occupational, environmental and lifestyle exposures of parents on the occurrence of infant leukemia is studied. Infant leukemia diagnosed within the first 6 months of life occurs at a frequency of around 1 case in 50.000 live births and shows a consistent genomic defect involving the 11q23 band (the MLL gene). These rearrangements are considered to offer circumstantial evidence for an environmental factor being involved and compelling evidence exists that the particular mutation is acquired in utero.

METHOD

In a series of 26 cases diagnosed between 89 and 97 in 7 European countries, parental exposures before and during pregnancy were studied. Information was gathered via face to face open interviews.

RESULTS

During the first half of the index pregnancy far most of the mothers had experienced considerable exposure to several factors, some of which known or suspected to have a leukemogenic potential. Fifteen (15) out of 26 mothers resided in environments contaminated by mold growth at the time of pregnancy or in the year preceding conception. In at least 8 of them the contamination was important and/or construction and repair work or intensive cleaning up was undertaken during pregnancy. One of these houses could be investigated: 6 years after the pregnancy of the index child during which important renovation works were performed in order to solve the severe humidity problem, one colony of *Stachybotrys atra* was isolated.

CONCLUSION

The high number of mothers reporting to have lived or worked during pregnancy or in the year preceeding conception in environments contaminated by mould growth suggests that exposure to mycotoxins should be considered in research for etiologic factors in infant leukemia. Epidemiological and experimental data indicate a possible link between some mycotoxins and severe effects on the hematolymphopoietic system, including leukaemia. More in particular the trichothecenes group of mycotoxins, produced by moulds like *Stachybotrys atra* might be incriminated. Concern about the particular sensitivity of infants to these mycotoxins was recently raised due to the association with acute pulmonary hemorrhage in Cleveland, Ohio.

MICROBIAL CONTAMINATION LITIGATION: EXPERT WITNESSES AND SCIENTIFIC EVIDENCE

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KEY WORDS:

expert witness testimony; consultants; attorney-client privilege; scientific evidence; admissibility; microbial contamination litigation; Frye; Daubert; Centex.

INTRODUCTION

Experts perform a crucial function in our justice system: to educate the jury and the court about the nuances and gray areas of a case. Expert witnesses benefit a party to a lawsuit by educating the party's attorney, helping to evaluate the case, and assisting in the determination of which elements of the case require proof by other expert witnesses.

Lawsuits regarding microbial contamination revolve around the expert witnesses. These cases are often little more than "battles of the experts," and can be won or lost on the effectiveness of the experts. Effectiveness requires preparation. A prepared but under-qualified expert may prevail over the more qualified but under-prepared expert.

This article addresses the role of an expert witness in the litigation process, especially in microbial contamination cases. It is written from the perspective of California law, and is not intended to be legal advice.

WHAT IS AN EXPERT WITNESS?

One definition of "expert witness" is an authority on a particular issue or subject matter who is willing and able to testify at trial. The expert must generally be identified in advance of the trial if another party serves a demand for exchange of expert witness information. However, if no such demand is served, the expert can testify and surprise the unwary litigant.

There are a number of key characteristics that distinguish expert testimony from lay testimony. Unlike lay witnesses, experts are permitted to and are expected to testify as to their opinions, and (with a few exceptions) may testify as to an ultimate issue to be decided in the case. Experts are permitted to answer hypothetical questions. Experts are also allowed to get paid to testify.[1] Unfortunately however, the nature of the payment system creates an environment ripe for abuses.

Expert witnesses can generally testify: (1) Where qualified; on (2) Subject matter sufficiently beyond common experience; to (3) Assist the trier of fact regarding (4) Matters perceived or

made known to the expert if (5) The testimony is based on matters reasonably relied upon by experts in forming such opinions.

In a toxic mold case, it is not unusual for each side to designate a dozen or more experts, in fields including construction, engineering, architecture, industrial hygiene, microbiology, remediation, environmental medicine, occupational health, allergy, immunology, toxicology, psychology, neuropsychology, neurology and many others.

Expert vs. Consultant

Another unique characteristic of these witnesses is that their character may change through the course of litigation, i.e., they may undergo a metamorphosis from "consultant" to "expert." In essence, a consultant is one who acts behind the scenes to advise the lawyer about the case. Consultants do not testify. Would-be experts frequently act initially in the role of "consultants," only later being formally designated as an "expert" to give opinion testimony at trial. The difference between the two is very important.

The communications between the attorney and a consultant are generally protected by the attorney-client privilege and the attorney work product doctrine. The rationale for discouraging the disclosure of attorney work product is to prevent a litigant from riding on the coattails of the opponent or taking advantage of the efforts of the adversary. In its purest sense, the work product doctrine is designed to protect the opinions, conclusions and strategies of a lawyer.

However, communications with an individual designated as an "expert" witness are not protected, because an expert is a witness who will testify at trial, so everything is "fair game." In other words, there is no legal confidentiality between the lawyer and the designated expert. All communications between the lawyer and the expert (including those made during the consultant phase) must generally be disclosed to the adversary on request. Attorneys should discourage experts from "sterilizing" their files, and encourage experts to follow the same ethical rules imposed upon lawyers. This is the type of conduct that will be necessary to begin rebuilding the badly scarred reputation of our justice system, and improving the confidence of the public.

A typical approach is for the attorney to initially hire the individual in the capacity of a consultant with an eye toward ultimately designating the individual as an expert witness. If there is any possibility the attorney will ever want to call the individual as an expert witness, the attorney should use special care in communicating with the consultant, especially in written communications. The attorney should reveal everything to the consultant that is relevant to the consultant's field of expertise. However, the information should be presented in a format as objective as reasonably possible, i.e., the lawyer should try to avoid tainting the expert's opinion with slanted statements about the case.

The expert is responsible for educating the lawyer about the field of science. This is time-consuming. Many lawyers who litigate microbial contamination cases have wandered into them unknowingly, trying to use the same approach they would use for other types of cases, e.g., personal injury. However, most other areas of law do not require the intensive involvement of experts that is typical for a microbial contamination case. If the lawyer works the case like a car

accident case (with the experts taking a back seat to the other witnesses), there may be a rude awakening.

The attorney will typically hire an individual in the capacity of "consultant" to assess the case, and render an opinion. If it appears that the individual's testimony would be helpful at trial, the consultant may be retained as an expert witness, to be designated later as an official player on the litigation team.

The proceedings herein illustrate that assessments of indoor environments may give rise to a variety of different interpretations. As stated above, a primary function of the expert is to clarify the gray areas of a case, and a party is entitled to seek well-reasoned expert opinion favorable to his or her position in the case. This is not unethical or underhanded. It is no different from trying to select jurors more likely to find in your favor.

The Litigation Team

Although the experts in microbial contamination cases are key players on the litigation team, being a part of a litigation team carries with it the duties and responsibilities one would otherwise assume in being a member of any other kind of a team. The purpose of a team is to benefit its owner. Ideally, the growth and advancement that the players enjoy as a result of being on the team should be nothing more than a mere consequential result of the success achieved in their efforts to benefit the owner.

In the legal context, the owner of the team is not the lawyer, it is the lawyer's client. Even though the expert's agreement may be with the lawyer, and the checks are signed by the lawyer, the expert is really employed by the lawyer's client—not the lawyer. Hence, the expert should always ethically act with the client's best interest at heart. The client's agenda (or well-being) is theoretically the one and only agenda worthy of recognition.

To be an effective member of a litigation team, each player (lawyers included), should leave the egos and agendas at the door, and do his or her best to ethically and honestly serve the best interests of the client. The well-oiled litigation machine operates with much internal collaboration and cooperation. The expert can attempt to change the lawyer's opinion about the case, but it is arguably improper for the lawyer to try to change the expert's opinion. That opinion should be formed in an objective vacuum.

However, the expert should be open to advice from the lawyer as to the style or manner in which the information is presented, because the lawyer's job is to formulate the strategy for the presentation of the case. In other words, the expert should respect the fact that the lawyer is the team captain—not because the lawyer's knowledge of the science is superior (certainly it is not), but because the litigant has chosen the lawyer to be the director of the show. On the other hand, this is not to say that the expert should sit back idly while the lawyer steers the ship off the edge of a cliff.

A good film producer is probably somewhat knowledgeable about writing, acting, cinematography, sound and lighting—but is an expert at none of those. Similarly, the lawyer will hopefully have a broad yet concise theme for the case that includes an understanding of how the various disciplines of expert testimony fit with the rest of the evidence of the case to comprise one complete presentation. The actors need to trust the producer to steer the ship in a

worthwhile direction, but will offer appropriate warnings if the ship wanders too far into dangerous waters.

A well-organized litigation team in a microbial contamination case should probably have a "lead expert" who will guide the technical side of the case, refer the attorney to preferred experts in related fields, and whose opinions are given special weight by the lawyer during the formation of the case strategy.

"More Data, More Data"

In order to arrive at a well-reasoned opinion that will survive the scrutiny of cross-examination, the expert must review and be familiar with the content of all pertinent evidence, reports, correspondence, laboratory data, depositions and other evidence from the case. It is better to review too much than too little. The lawyer can reasonably be expected to comply with the expert's requests for information, and should never mislead the expert or conceal important facts. Aside from being dishonest and unethical, this would surely backfire on the lawyer when the facts are sprung on the expert during cross-examination.

Obviously, the experts should form their opinions based on the actual evidence of the case, rather the attorneys' statements or arguments. The expert must be prepared to cite a particular portion of the official record of the case for all facts relied upon in forming the opinion.

The information upon which the expert relies may itself be inadmissible in court.[2] For instance, the expert can rely on hearsay, so long as the expert's testimony is not based "primarily" upon inadmissible hearsay.[3]

However, the materials relied upon by the expert must be of appropriate caliber for the subject matter. For example, periodicals relied upon must constitute the type of professional technical literature reasonably relied upon by experts in the field. In a case regarding lawn mower safety, a court can properly exclude articles from Reader's Digest, Today's Health, and Consumer Bulletin. Microbial contamination raises special admissibility issues, especially in Frye[4] jurisdictions.

In Centex-Rooney Const. v. Martin County,[5] a construction manager unsuccessfully appealed a final judgment of over $14 million, resulting from construction defects and revealed in a "sick building" case. The construction manager objected to the plaintiff's expert testimony which suggested the existence of a health hazard stemming from the toxic molds found in the buildings.

The Centex court conducted Frye hearings, and the construction manager argued that the studies linking health risks with toxic mold exposure were "not generally accepted in the medical community." The trial court disagreed, and the Court of Appeals affirmed, on grounds that the plaintiff's experts "testified about numerous publications accepted in the scientific community recognizing the link between exposure to the highly unusual toxigenic molds and adverse health effects."[6] "We find that the County met its burden under Frye of proving by a preponderance of the evidence that the basic underlying principles of scientific evidence were sufficiently tested and accepted by the relevant scientific community."[7]

PREPARING TO TESTIFY

THE GOAL

Your goal as an expert witness is to convince the jury you're right. Doing so without seeming partisan is an art form. You need to appear neutral as to the parties, yet steadfastly committed to your interpretations of the evidence and opinions of the science.

Good experts show that they are more prepared and more knowledgeable than the opposing expert. The conventional wisdom/stereotype among lawyers says that gray-haired warm fatherly-type experts are better received by juries. Good expert witnesses set a mood when they testify. In the inner sanctum of law offices, some attorneys fantasize about their experts setting a mood in the courtroom akin to Robert Young sitting in an easy chair next to a fireplace, wearing a robe and slippers, smoking a pipe, telling a story to his grandchildren as a golden retriever relaxes on the floor beside him.

Proper preparation requires the expert to review literature adverse to her position. The expert should strive to avoid surprise at trial. The expert who has not read adverse literature may be considered unprepared or incompetent by the jury. However, an expert generally cannot be questioned about adverse literature that the expert did not rely upon, consider or refer to in arriving at or forming the opinion.

SIMPLIFY, SIMPLIFY, SIMPLIFY

Experts should teach the jury...with plain English. Scientists who give expert testimony and use too much scientific gobbledy-gook have lost before they start. Technical jargon, although perhaps impressive at first, quickly becomes convoluted and ultimately boring, and as such, should only be used where absolutely necessary. When it is used, it should always be in conjunction with its plain English translation.

In other words, an expert should not address a jury in the same manner she would teach microbiology to her graduate students, or make a presentation at the Third International Conference on Bioaerosols. Most jurors probably do not know what a trichothecene is.

One especially effective technique to communicate esoteric scientific principles is through the use of analogies and metaphors from everyday life. For example, to illustrate the point that air samples can yield false negative results, one skilled industrial hygienist testifies that air sampling is like fishing: just because you catch no fish does not mean the fish were not there. Perhaps you had the wrong bait (media). This simile creates a lasting visual impression that illustrates the point beautifully and helps to keep the testimony interesting.

IMPRESSIONS

One legal philosopher, apparently frustrated with the way some jurors rely too heavily on impressions, explored the possibility of eliminating the jury system and simply using computers to decide cases. Certainly, a computer can be programmed with the speed limit on Main Street, so if the speed driven by Dan Defendant is entered, the computer would give the theoretically "correct" answer as to whether Dan violated the speed limit.

However, if the law says that a driver can never drive faster than is safe, how can the computer determine what is "safe"? The law must be versatile enough to resolve any imaginable situation. Accordingly, many of our rules of law contain purposely open-ended terms such as "reasonable," "equitable," "fair," "prudent," and the like. Complex situations require the weighing and balancing of many different facts and special circumstances. Computers might render queer results in such cases. This is why we have juries.

Computers cannot tell us what is fair; it requires analysis of too many factors. Computers cannot be programmed to exercise common sense—the cornerstone of our law. Since standards of conduct tend to vary culturally from one region to another, "common sense" in Los Angeles may bear little resemblance to that in Albuquerque. The skilled expert witness understands the dynamics created by the fact that in our legal system, decisions are made by humans, not by computers. Accordingly, quality of scientific evidence is only one part of the battle. A good impression goes a long way.

The three groups of "players" in microbial contamination cases—jurors, attorneys and experts—see cases in markedly different fashions. Each of these groups must learn to appreciate the perspectives (and impressions) of the other two groups; this will make a more efficient justice system.

For some jurors, the way in which the evidence is presented seems to take on a more important role than the quality of the evidence itself. Unlike computers, jurors have the ability to reason and make decisions about credibility and veracity based on their life experience. Life experience is probably the most important asset jurors bring to the courtroom. Of course, good science, logic and facts are important, but many cases—perhaps too many—are decided on emotion, passion, gut feelings, and sometimes, bias, prejudice and/or secret agendas.

Statements made by jurors after trials suggest that they have a tendency to decide cases based merely on their own personal dislike for a party, their lawyer or their experts, despite instructions from judges prohibiting this.

Lawsuits are all about impressions—in more ways than one. He who makes the best impression wins. Lawsuits arise because two parties have different impressions about an event, or a series of events. At trial, a lawsuit may be resolved as a result of a jury's impression about the parties' impressions. This is impressionism compounded. Much of what the jury hears has been diluted, or at least filtered, by the impression system of the witnesses who tell the story as they see it.

For instance, witnesses A and B, standing on the same street corner at the same moment, have different impressions of how a traffic accident occurred. The drivers of the two cars have different impressions about how the accident occurred. Similarly, the jury will decide the case based on its impressions—not just of the evidence, but of the lawyers, the parties, and the expert witnesses—especially in microbial contamination cases.

Lawyers are in the message delivery business. The lawyer must develop a cogent theme for the case,[8] and every piece of evidence should support an element of that theme. If the expert's opinions conflict with the theme, the expert should withdraw from the case, and do so early enough to give the attorney ample opportunity to locate and retain another expert. The courtroom is not the place for the members of the litigation team to air their differences. Their job

is to help the party who hired them, and they help no one by putting their respective agendas ahead of the interests of that party.

Numerous studies have suggested that juries retain a far greater percentage of non-verbal communication than verbal communication. Hence, factors such as an expert's reaction and tone carry an under-appreciated significance. Eye contact, facial expressions, hand gestures, fidgeting and other non-verbal cues may have a deeper impact than the results of a first-class research study. Juries have reported that these cues have given them the impression an expert witness is disinterested, arrogant or egotistical.

From the moment any witness gets out of the car in the courthouse parking lot, it is as if the witness is on stage. Seeing spinach in the teeth of a witness could have a greater impact on the impression of a juror than four hours of brilliant testimony.

Cross-examination

The opposing party has an opportunity to take pot-shots at an expert in the process which is the cornerstone of an adversarial legal system: the dreaded cross-examination. Depositions are essentially pure cross-examination.

There is seemingly little restraining the lawyer cross-examining an expert witness. Many of the rules that restrict a lawyer on direct examination do not apply on cross-examination. Provided the cross-examination does not reach into areas beyond the scope of direct examination, the lawyer has seemingly free reign to do as he/she pleases. It will probably seem as though the lawyer is taking bits and pieces of unconnected facts out of context in an effort to craft a misleading tale.

However, any witness being cross-examined should concede the obvious and the unimportant, and answer with one word answers where possible—resisting the temptation to explain the answers. Explanation can open the door to new and potentially damaging areas. Every word out of the mouth of the witness is fair game for follow-up questioning. When the lawyer who retained the expert conducts "re-direct," the witness will have the opportunity to explain any misleading responses or answers to questions asked out of context.

The law disapproves of the harassment of any witness. However, the threshold of what judges will deem "harassment" varies based on the age, education and strength of the witness, and virtually any other reasonably relevant factor under the circumstances. Not surprisingly, an expert witness with ten years of experience testifying, and who is charging $450 per hour will probably receive little, if any, sympathy from a judge when aggressively attacked by the opposing attorney. The savvy expert will expect leading questions pregnant with accusation, and will bite their lip to avoid getting angry at offensive assaults on the expert's integrity. Some attorneys want opposing experts to lose their temper, because if they do, the expert loses the non-verbal battle.

Depositions

Expert depositions, although disallowed in some states, are often the most important events in microbial contamination cases, due in part to their far-reaching impact on settlement negotiations. Most lawsuits settle before trial, primarily because trial is expensive and risky. The settle-

ment value of a case (and hence, its outcome) is often determined by the strength of the deposition testimony of the experts.

There are a number of different ways to depose an expert. Some lawyers go right for the jugular, challenging the opinions of the expert, and holding nothing back as they attempt to tear the expert apart. Others limit the inquiry to the expert's opinions and what they are based on, opting not to tip their hand before trial. This latter group hopes to effect a surprise attack on the expert at trial.

Deposition transcripts are used to attack trial witnesses with their own words. Hence, all witnesses should carefully review their deposition transcripts before testifying at trial, in an effort to be crystal clear on factual details, and to anticipate the strategy of the examining attorney. In sum, experts should not underestimate the importance of depositions. They should prepare for depositions just as they would for trial.

CONCLUSION

Lawsuits rise and fall because of impressions. Cutting-edge law follows on the coattails of cutting-edge science. The new developing area of microbial contamination litigation promises to be a dynamic area of practice--for both experts and lawyers, who can make lasting impressions on juries about novel scientific concepts. Due to their complexity and technicality, these cases cry out for the testimony of well-prepared specialists, who are not only expert scientists...but expert communicators.

END NOTES

[1]However, the savvy expert testifies that he or she is not being paid to render a specific opinion.

[2]See, e.g., Robertson v. Union Pac. R. Co., 954 F.2d 1433, 1435 (8th Cir. 1992).

[3]Hearsay is an out-of-court statement offered to prove its truth. The exceptions to the "hearsay is inadmissible" rule are so broad and numerous, they almost swallow the rule. Hence, there can be a large body of "admissible hearsay."

[4]Frye jurisdictions require the proponent of evidence generated through the use of new or novel scientific techniques to show that the technique is "generally accepted" in the scientific community. This presents special issues in microbial contamination cases, where the science is arguably in a state of flux and certain testing techniques may be controversial. In contrast, Daubert jurisdictions have a lower threshold; the evidence will generally be admissible if the proponent is able to establish scientific "validity." Consequently, if good scientific practice was followed, but the technique is not generally accepted in the scientific community, the results would probably be admissible in a Daubert jurisdiction, but inadmissible in a Frye jurisdiction.

[5]706 So.2d 20 (Fla.App. 4 Dist. 1997)

[6]Id at 26.

[7]Ibid.

[8]This can be difficult in a microbial contamination case.

FIELD REPORT: INITIAL MICROBIOLOGICAL ASSESSMENT IN FOUR NEW CONDOMINIUMS

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ABSTRACT

A risk assessment was performed at the request of the owners of four new condominiums in a condo complex in central New Jersey. The owners had a history of complaints starting immediately upon occupancy and believed that these health effects could be due to exposure to airborne fungi. To assess airborne, viable fungal exposure levels and the resulting potential for adverse health effects, an initial assessment was performed.

After interviewing occupants, and conducting a cursory visual inspection, air, surface wipe and bulk samples were collected and analyzed. The HVAC systems of the units were briefly inspected, and very preliminary analyses of moisture sources and pollutant transport were completed. Occupants also completed "a" questionnaire to evaluate reported health effects.

Inspection procedures, test results, and evaluation of health effects questionnaires indicated strong correlation between observed conditions and occupant health problems.

It was concluded that uncontrolled moisture has resulted in significant microbial amplification which led to the decision to recommend evacuation of the units. Litigation is proceeding. All sources of moisture need to be identified before any appropriate remedial strategy can be offered. These units may have to be rebuilt.

KEY WORDS:

Actinomycetes, Aspergillus, Penicillium, Bioaerosols, Moisture Intrusions, NIOSH Questionnaire, toxigenic fungi, Gram negative bacteria

INTRODUCTION

The initial survey was conducted on October 5, 1996 by the authors. The scope of the survey consisted of a visual inspection of the interior and exterior of the residences, interviews with the occupants and sampling for viable bacteria and fungi.

Initial sampling results indicated microbial proliferation and fungal contamination with the potential to affect resident health. The source(s) of the moisture responsible for the fungal amplification were not evident during this initial assessment.

It was not certain if these residences could be remediated in an economical fashion. As noted, the first issue to be resolved was to determine the source(s) of moisture which were responsible for the microbial growth. If these sources could be successfully identified and corrected, it

would then be necessary to determine the extent of the fungal and bacterial contamination in the condominium units. This would include addressing the facilities themselves and also the interior furnishings.

The original report advised: "Until such time as the levels of fungal contamination noted in the initial sampling results can be reduced, occupants who have had adverse health impacts or who feel ill when in their homes should spend as little time as possible in the condominiums. It is also recommend that all occupants be evaluated by a qualified Occupational and/or Environmental Health physician (M.D.)."

This paper outlines the observations noted during the initial site visit, microbiological sampling methods and results. Also included in the report is a discussion of the sampling results and the potential health effects from the microorganisms identified during the sampling. Several conclusions and recommendations were offered for consideration and further investigation.

Outlined below are those observations made during the brief building inspections and comments from building occupants regarding air quality in the building. At the initial meeting with the occupants the following points were made:

1. Health effects which are believed to be related to the air quality were noted by all the occupants within a short time after moving into the four condominium units studied. These effects included shortness of breath, chest tightness, swollen throat, headaches, loss of coordination and confusion, fatigue, sinus infections, ringing in the ears, eye irritation, congestion in sinuses, asthma attacks, bronchitis, and swollen joints. They also noted that these effects seemed more profound in the lower levels of their homes.
2. The residents noted problems with achieving acceptable temperature levels in the units. Although the air conditioning system was cooling the lower levels sufficiently, there was anecdotal evidence suggesting that the system was producing cool but moist air. In addition, occupants related that the system was not cooling the upper levels properly.
3. Visible mold growth was evident in 3 of the four units. A musty, moldy odor was evident in 2 units while a slight sewer odor was noted in one unit.
4. The units were all depressurized with levels ranging from 8 to 15 Pascals negative with respect to the outdoors. Additionally, radon levels were as high as 70 pCi/liter.

The condominiums investigated are the first to be occupied in the complex which was being developed. There was on-going construction work on other units in the complex, all of which are at a higher elevation than the units in question. It was noted during a brief inspection of the site that storm water system was blocked off in certain locations. Numerous puddles and pounding of rainwater was seen in the construction areas. This may indicate low soil permeability since at the time of the site visit the weather had been relatively dry in recent days.

The storm water collection basins are located to the southeast of and adjacent to the condominium structure where the 3 of the 4 units evaluated are located. It was noted that the upper retention basin was situated at an elevation higher than the lower level of these units.

The roof gutters are piped to an underground drain located at the front of the units but their destination could not be determined visually. A leaky valve in the back flow prevented in the sprinkler room was noted and did not appear to properly drain out of the building.

It was reported that the sanitary system had a blockage which resulted in an overflow of drain water in Unit #5 on two separate occasions.

The other condominium is located in the first unit up from the other three and is equipped with a wet sump in the floor of the lower level.

METHODS

Microbiological Testing

Microbiological testing included the collection of one surface wipe sample and 3 air samples for viable organisms in the lower levels of each of the four units. No outdoor reference samples were collected since the occupants did not desire to spend any more in analytical fees than necessary.

An additional surface wipe sample was collected from a stairway tread in an unfinished unit adjacent to the one of the units. There was significant visible mold growth on the unfinished wood surfaces. All samples were incubated and cultured to determine microorganisms present.

Air samples were collected using an Andersen single stage impactor running at 1 cubic foot/minute for 3 minutes. Sampling media consisted of malt extract agar (MEA), a general purpose agar, and cornmeal agar (CMA) to try to isolate *Stachybotrys chartarum*, if present. The tests for *Stachybotrys* were completed at the request of the occupants who had read about this fungus in recent newspaper articles about a library in Staten Island, N.Y. Tryptic soy agar (TSA) was used for bacterial samples.

Wipe samples were collected using sterile swabs with a moisturizing ampule. Surfaces to be tested were wiped with the wet swab.

Additionally, one bulk sample of the carpet underpad near the secondary bathroom was collected in the one unit which was "decontaminated."

Analysis was completed by P&K Microbiology Services of Cherry Hill, NJ, by Chin S. Yang, Ph.D. The results of the sampling for microbiological assay are detailed in the tables in the Appendix to this report.

RESULTS AND DISCUSSION

It is important to base risk assessments and interpretation of sampling results for bioaerosols (air sample results) on the indoor/outdoor ratios and the concept of reservoirs, amplification, and dissemination.

Reservoirs are areas conducive to microbial growth which provide the conditions necessary for such growth including nutrients, appropriate temperatures and water. Amplification is the growth of these organisms, and dissemination refers to mechanisms which can spread the organisms throughout a building.

There were obvious, and likely not so obvious, reservoirs in these homes as evidenced by the visible mold growth (amplification) and overall plate counts. Surface moisture must be present

to promote and sustain the fungal amplification. Dissemination mechanisms include the ventilation system and occupant activities.

As noted in the laboratory report, there were high fungal and bacterial levels detected in the air samples in all the condominium units. Plate counts were typically unable to be determined since the plates were overloaded (>4711 CFU/m^3). The dominant fungi in the samples were *Penicillium* and/or *Aspergillus versicolor*. *Trichoderma*, frequently associated with water damaged wood or paper products, was noted in two air samples.

Environmental bacteria, including Actinomycetes and *Gram negative bacteria*, were the dominant bacterial species identified in the air. These environmental bacteria indicate the presence of water or wet conditions.

The bulk sample showed moderate fungal and high bacterial levels. As with the air samples, *Aspergillus versicolor* and *Penicillium* were the dominant fungi and *Gram negative species* were the dominant bacteria.

Surface wipe samples yielded elevated fungal and bacterial levels. Corresponding with the air and bulk samples, *Aspergillus* and *Penicillium* were the significant fungi found in wipe samples. *Bacillus* and *Gram negative bacteria* were bacterial types identified on building surfaces.

Surface wipe samples are notable for the presence of *Aspergillus versicolor*, other *Aspergillus* species and *Penicillium*. Again, these fungi have the potential to produce mycotoxins. The wipe samples collected in the unoccupied unit was also dominated by *Penicillium* with *Aspergillus* also identified in the sample. These results are similar to those in the occupied units.

These results indicate the presence of certain toxigenic fungal species. Potentially toxigenic species present in these homes include *Penicillium*, *Aspergillus* species including *Aspergillus versicolor*, *Cladosporium*, *Trichoderma*, *Epicoccum*, and *Alternaria*.

Gram negative bacteria were noted as the dominant bacteria in the bulk, wipe and several of the air samples. *Gram negative bacteria* are environmental bacteria as opposed to human associated bacteria such as *Staphylococcus* or *Micrococcus*. Human associated bacterial species would normally be expected to dominate the fungal spectrum in an indoor, human-occupied environment. *Gram negative* dominant results usually indicates water related conditions.

Actinomycetes was also noted in the bacterial samples. Its presence is indicative of unusual reservoirs (growth sites) or water contamination.

The relationships between health effects and exposure to microorganisms must be determined through a combination of medical, epidemiologic, and environmental evaluation. As previously noted, the environmental assessment identified visible mold growth in all the residences (except the unit which had been recently cleaned) and high airborne bacterial and fungal levels as well. Also, the symptoms described by occupants are consistent with exposure to elevated airborne levels of fungi and bacteria and the species of organisms isolated. Please note that medical and possibly epidemiological assessment are also part of a comprehensive overall evaluation as well.

REVIEW OF HEALTH EFFECTS QUESTIONNAIRE AND INTERVIEWS

In an effort to determine the nature of the perceived IEQ problems, the five occupants were interviewed and also asked to complete a questionnaire. It was modified from the EPA document *Introduction to Indoor Air Quality - A Reference Manual.* Symptoms listed on the EPA document can relate to a number of issues. In a recent paper, the National Institute for Occupational Safety & Health (NIOSH) grouped symptoms into the following three categories.

multiple respiratory symptoms: shortness of breath, cough, chest tightness, and/or wheezing

multiple atopic symptoms: sneezing, itchy/irritated eyes, stuffy/runny nose or sinus congestion

multiple SBS symptoms: headaches, sore or dry throat, nasal congestion, fatigue, or irritated eyes

To be classified into a particular group or groupings, NIOSH applied the following evaluation criteria:

Multiple respiratory - 3 of the 4 symptoms

Multiple atopic (allergic) - all three symptoms

Multiple Sick Building Syndrome - 3 of 5 symptoms

The summary of responses to the symptom checklist indicate that all 5 occupants who completed the questionnaire and who were interviewed met the criteria to be classified into *all three categories*, namely the multiple respiratory symptoms group, the atopic group, and the SBS group. Symptoms reported are consistent with the presence of allergens and sensitive occupants (atopic and respiratory groups). Particularly cogent responses include the following:

1. Each occupant feels that their symptoms improve upon leaving their homes and that the majority of their health problems did not begin until they moved into their current homes.
2. Residents were advised by the condominium management company that the reason that mold was growing in their homes was that the air conditioning systems were not operating all the time and that problems would abate if the air conditioning was operated constantly.
3. Occupants daily living routines have been seriously impacted as evidenced by two factors. These include the fact that some residents simply have chosen to stay in their homes as little as possible due to the health impacts they feel are caused the indoor environment in their homes. Also, a large number of health effects noted by the residents were identified as frequent (more than once a week) or "severe" requiring a major change in their daily routine.

CONCLUSIONS AND RECOMMENDATIONS

The initial report noted "Until such time as the microbiological contamination is eliminated, these homes should not be occupied by any persons who feel that their health is being impaired or if, when in their homes, the occupants continue to experience the health problems previously described."

It is believed that the microbial contamination problems are being caused by high moisture levels due to either wet building materials which have not been dried out, design or construction defects, or external or internal water source(s) and pollutant transport mechanisms.

In the former case, it is not uncommon for building materials to become wet during construction. However, if the design or construction does not allow for the building to dry itself out then interior moisture conditions will persist.

Since the condominium occupants were advised to continually operate their air conditioning units, it would appear that the management company believed that this would "dry-out" the building. It is unfortunate that this only exacerbated the problem. The air conditioners are not designed or sized for dehumidification purposes.

It appears that their operation actually resulting in increasing the relative humidity levels in the lower (ie. cooler) areas of the condominiums.

Cooling these areas and their interior building surfaces without removing interior moisture effectively increased the relative humidities in these units. Without the introduction of warmer, drier outside air (or using a dehumidifier as was employed in one of the units), the cool and moist conditions in the lower levels of these units provides a good environment for fungal growth.

The latter assumption regarding an external source of moisture would include water inflow from an improperly draining sanitary sewer system, the storm water retention basin and related underground piping or high ground water related to site conditions or on-going site work. It is recommended that these systems be inspected for proper functioning.

Once sources of moisture and water are determined, total extent of microbiological contamination in each unit must then be determined. When this assessment is completed, a remedial strategy can be planned accordingly. Any microbiological decontamination work must be conducted under *controlled conditions* following stringent protocols which will protect workers and future occupants. Further, appropriate post-remedial clearance criteria must be established and attained prior to re-occupancies of the affected areas of the condominium units.

1. Occupants were advised to "close their windows and keep the A/C running continuously."
2. The situation deteriorated to the point that all occupants moved out of the units due to persistent health impacts and lack of resolution on the part of the builder who refused to acknowledge a moisture problem existed.
3. Lawsuits and counter lawsuits have been filed. One occupant has parked her car in front of her unit with signs and skull and crossbones pictures taped top the windows.
4. The builder's solution was to place dehumidifiers in the units and to wipe walls with a disinfecting solution of a bacteriostatic product. He then advised the occupants to run the dehumidifiers constantly.

   Nothing was done relative to the moist carpeting or to truly evaluate all sources of moisture impacts. He did not acknowledge any other impacts from moisture other than high RH in the units. Air tests by the consultant hired by the builder revealed outdoor levels less than indoor levels with no speciation of the samples. Based on this test, conducted in early March, the consultant concluded that the units were able to be occupied by the owners.
5. It is felt, based on a cursory evaluation, that elevated RH levels are the least of the problems in these units. Visual inspection of other units under construction revealed that no polyethylene sheeting was placed over the foundation dirt prior to pouring the concrete basement floor and that the term "vapor barrier" was unknown to the builder.

6. Reports from the builder's consultant concluded that there were few, if any problems in these units that dehumidification would not solve. Data supplied with the report included plate counts for bacteria and fungi which were added together. There was no speciation or other efforts to identify dominant species. Indoor counts exceeded outdoor counts.

LEARNING FROM *STACHYBOTRYS CHARTARUM:* HOW TO FIND HIDDEN MOLD IN BUILDINGS.

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ABSTRACT

An open and multidisciplinary approach is needed to make a complete, fast and efficient diagnosis of hidden mold problems in buildings. A typical case study involving a *Stachybotrys chartarum* contaminated building will be used to illustrate the many critically important steps that are required to make your diagnosis a success story. This four story office building, located in Montréal (Québec) experienced extensive water damage from the 1998 ice storm. After partial evacuation prompted by health officials, numerous remaining occupants complained of respiratory and other symptoms.

Visual inspection of the premisses and their ventilation showed extensive water infiltrations from the roof top ventilation units and from the lower part of the exterior walls. This abnormal water activity caused fungal contamination with *Stachybotrys chartarum* and *Ulocladium* on surfaces with a total visible surface of around 1.5 square meters. Deposited dust in carpets, wall board and ventilation units was heavily contaminated with millions of cfus per gram of Gram positive bacteria, yeasts and *Trichoderma viride.*

Remaining occupants left the building and their symptoms were either alleviated or disappeared completely.

Remediation was done according to the New York Department of Health protocol for *Stachybotrys chartarum* contamination. Upon opening of wet walls, an additional surface of 10 square meters was found with heavy *Stachybotrys chartarum* mold contamination. This finding shows that the vast majority of moldy surfaces was hidden and would not have been visible without opening the structure.

INTRODUCTION

THE MOLD PARADIGM: BETWEEN "TOO MUCH" AND "NOT ENOUGH".

In the field of mold control in buildings, every step forward is the catalyst for renewed controversy.... and headaches for consultants. What is "not enough" in the eyes of certain people is already "too much" in the eyes of others.

In fact, this situation is not surprising: in the last thirty years, there has been a major change in our way of life. After the energy crisis, a growing concern about keeping energy costs as low as possible gave rise to a new generation of houses and buildings, with minimal fresh air intake and maximal thermal insulation. In parallel, numerous unprecedented waves of budget cut-

backs decreased the quality level of maintenance in buildings. In consequence, mold contamination problems due to uncontrolled water activity in buildings and in HVAC system, especially those with porous duct insulation, are now more and more frequent in these "air tight" but "water leaky" constructions.

Public Works and Government Services Canada, after investigating 95 office buildings between 1987 and 1994, have discovered that, in 21 % of these buildings, microbial contamination was the major contributing factor to the low quality of indoor air.

In addition to microbial contamination in buildings, the overall load of pollutants is rising in water, soil and air. The presence of chemicals is unavoidable in every aspect of our everyday life: food additives, pesticides and drugs, etc... This situation has certainly contributed to the sharp rise in allergies and hypersensitivity problems in our population. People are therefore more sensitive than before to mold contamination in their indoor environment.

For all these reasons, and in that context, it is misleading to refer to *Stachybotrys chartarum*, or other mold contaminants in buildings, as "an old problem that is now given exaggerated attention". Mold is ubiquitous, and has been for millions of years, but its impact in indoor environment is now much greater than it ever was. As building consultants for many years, we have been working to solve microbial contamination problems for people, in a way that restores their quality of life and doesn't make remediation a heavier burden than the mold problem itself. On the other hand, it must be kept in mind that incomplete remediation will leave people with the old problem coming back again... All our experience has been built on the fragile equilibrium between "too much"and "not enough". The following case study illustrates such a situation and how we dealt with it.

CASE STUDY

In search of the hidden mold.

After the January 1998 ice storm, administrators of a Montréal office building hired us to make a complete microbial evaluation of the premisses. Five occupants, out of 33, started to complain of asthma, flue like symptoms, and severe headaches after the ice storm. Local health officials (CLSC) prompted evacuation of the third floor.

Building history.

The four level (three floors and a basement) office building, built in the fifties, with a total surface of 20 000 square feet, has an exterior brick cover and a concrete basement. Five roof top HVAC units are installed on the flat roof. Thermal insulation and fire retardant properties are provided by asbestos amosite fibers on the H beams of the structure. There has been some water leaks mainly from the roof but also from the basement, with a major worsening of the situation after the January 1998 icestorm.

Health Investigation.

A medical doctor, with experience in this field, after an informational meeting with the occupants to assess the situation, concluded that at least 24 persons out of 33 were experiencing

symptoms that could be associated with indoor microbial contamination. Symptoms diminished or altogether disappeared when the occupants left the building.

ON SITE INVESTIGATION.

OUTSIDE :

Inspection of the HVAC roof top units has shown that they were very old, with inadequate maintenance, no access to the drain pans and that 10 inches of ice accumulation from the ice storm caused water infiltration and leaky joints all around the units, allowing large amounts of water inside the building.

FIRST, SECOND AND THIRD FLOORS :

Observations :

Mold odor on the second and third floor.

Wet carpets removed on the third floor.

Obvious traces of extensive water infiltration from the roof, with water buckets in many places, especially near the ventilation ducts.

Leaky surfaces on walls and ceilings covered with plastic to prevent water dripping.

Surface sampling on adhesive tape :

Fungal growth was found on the lower parts of certain walls, with the presence of visible *Stachybotrys chartarum*, on approximately 10 cm^2 and *Ulocladium* on approximately 0.5 m^2.

Fungal growth on carpet glue with 0.1 m^2 of *Stachybotrys chartarum* and 0.5 m^2 of *Ulocladium.*

Total visible fungal growth on a surface of approximately 1.5 m^2, mainly on the third and second floors, nearest to the roof.

Bulk dust sampling :

Dust sampling showed the presence of large amounts (over 1 million cfus per gram of dust) of *Trichoderma viride*, yeasts and Gram positive bacteria in the electrical wall board units of the third floor.

Carpet dust showed more than 12.5 millions cfus per gram of the same microbial distribution on the second floor and a million colonies per gram of Gram positive bacteria on the first floor.

Lesser amounts of microbes, with the same distribution, were found in HVAC system # 4, on the third floor.

Finally, the roof top unit of HVAC system # 1, for the first and second floor, was heavily contaminated with more than 5 millions cfus of *Cladosporium* per gram of dust.

BASEMENT :

Many sources of water infiltrations were present, but less extensive than roof leaking. The lower exterior brick cover was buried in the ground to level the outside area near the walls. This result-

ed in water infiltration inside the basement from the porous bricks, and small surfaces of visible *Penicillium*, on less than 0.1 m^2.

Remediation

Building evacuation :

The investigation did not show extensive surfaces of mold growth but it did show extensive abnormal water activity. The "not enough" attitude would have prompted us to recommend a stop to water activity from the roof and basement... and hope for the best. Simple cleanup of the water infiltration areas, without intensive inspection of the internal wall and ceiling structures would have been enough, because of insufficient evidence of mold growth.

The "too much" attitude would have prompted us to start panicking about the presence of the "deadly black mold" even on small surfaces, make unnecessary supplementary lab sampling and analysis, and lose credibility.

Even in the absence of large visible surfaces with mold growth, extensive water activity can cause hidden fungal contamination involving the building structure. Furthermore, dust sampling revealed contamination in many areas of the building, and occupants reported health symptoms. For these reasons, we recommended to evacuate the second floor and stop water infiltration by the roof in priority. The building administrators decided to evacuate all the building and we organized the microbial decontamination of the office equipment that was transferred in another building. After the occupants left the building, health symptoms either diminished or stopped entirely. Other recommendations followed, including the removal of asbestos and the fungal decontamination of all the premisses.

Asbestos abatement and microbial decontamination protocol :

Since work had to be done on the structure, including asbestos covered beams, asbestos removal as well as microbial decontamination were included in detailed quotations. Demolition, asbestos abatement and microbial decontamination were handled by contractors under our supervision. Asbestos abatement procedures followed federal and provincial regulations. Microbial decontamination procedures were guided by New York Department of Health, Public Works, Health Canada and Canadian Mortgage and Housing Corporation recommendations. A special appreciation goes to Dr Philip Morey, whose work and publications are invaluable in establishing confinement levels and strategies on contaminated sites.

Discovering the hidden mold :

After the retrieval of all the HVAC units, the roof was completely redone. Asbestos confinement and HEPA filter air extraction devices were put in place first on the third floor to control the removal of the fungal contaminated materials, including all the air ducts, than on the second, first and basement floors. Secondly, the asbestos fibers were taken away followed by the floor decontamination with a blast track machine. Finally, all the surfaces in the building were vacuum cleaned and wiped with a chlorine bleach solution.

As remediation proceeded, opening up of the wet structures revealed the presence of large surfaces of hidden mold growth, near the leaky ventilation ducting, where water infiltrated from the roof. This situation was not obvious during the initial walk through.

With the "not enough" attitude, not opening the structures, the mold would not have been discovered. But extensive water activity and health complaints from occupants called for opening up of the structures. This decision was indeed the right one:

a total hidden surface of more than 10 m^2 was contaminated by *Stachybotrys chartarum* mold growth, mostly in the bathrooms and the mechanical rooms.

CONCLUSION

First, trust the occupants: if there are complaints, there is a cause, even if it's not obvious. Find it! Second, a thorough inspection of the premisses and their ventilation is mandatory. Keep in mind that mold needs water and porous organic materials that can be hidden in the structures and not directly visible. Almost any building material can harbor fungal growth, if the available water activity is sufficient. Use the proper tools to find it. Third, if sampling is needed, be versatile: no sampling method is perfect. Adapt the sampling strategy to the situation and use an accredited mycology lab to count and identify fungi in your samples. Bear in mind that you could be dealing with toxigenic mold and, depending on the extent of the contaminated surface, take proper action to keep occupants away from it and protect their health. Finally, after putting together all the data gathered in the field, always communicate it in a simple, straightforward way, with an affordable step by step remediation plan: don't forget that people want to get rid of their problem, not make their lives more complicated.

CONCENTRATIONS OF AIRBORNE VIABLE SPORES OF FUNGI AND ACTINOMYCETES IN MOLD-DAMAGED OFFICES AND SCHOOLS

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ABSTRACT

Some Finnish guidelines exist for acceptable levels of fungal spores in indoor air in mold-damaged dwellinghouses. The object of the present work was to assess if the same guidelines are relevant for mold problems in office and school environments. Air samples (n=48) were collected in winter by using a six-stage impactor from 18 mold problem office and school buildings.

The concentrations of airborne fungal spores and actinomycetes were low in severely damaged offices and schools. The geometric mean (GM) of viable fungal spore counts in indoor air was 31 cfu/m^3 in office buildings; in schools it was 97 cfu/m^3 in rooms with moderate water-damage and 132 cfu/m^3 in rooms with severe damage. The GM of viable actinomycete counts were between 3-5 cfu/m^3 in offices and schools in rooms with moderate damage, while the counts were 10 cfu/m^3 in severely damaged rooms. The results indicate that the Finnish guidelines for levels of airborne fungi in dwelling-houses are not relevant for office environments.

KEY WORDS

fungal spores, indoor air, school, office, moisture damages

INTRODUCTION

Moisture and mold damages are common in Finnish buildings (Koivisto et al. 1996). The association between moisture problems, mold growth and respiratory health disorders has been shown in many studies (Andrea et al. 1988, Bunekreef et al. 1989). In winter, fungal spore concentrations over 500 cfu/m^3 in indoor air are regarded to indicate an abnormal microbial source indoors and also possibility of health disorders based on Finnish guidelines for urban or suburban homes (Reponen et al. 1992). However, very little data has been published about fungal spore concentrations in office buildings. Aim of the present study was to examine the level of airborne fungal and actinomycete spore concentrations in offices and schools with moisture problems and to evaluate if the guidelines for the levels of airborne fungi in dwelling-houses are relevant also for office environments.

MATERIAL AND METHODS

Objects

In the present study, altogether 9 school and 9 office buildings located in Southern Finland were examined. The age of buildings varied from 11 to 70 years and they were equipped with supply and exhaust ventilation or mechinal exhaust only. Moisture damages were reported in all school buildings; the background information were given by the local health inspectors. Background information of moisture damages in office buildings were not known in advance.

Evaluation of construction damages

Construction damages were evaluated by an experienced architect and a building engineer. Evaluation was performed visually, noting the smell and measuring the moisture of surfaces. The areas of school buildings were classified into three categories according to the severity of moisture problems: "0 area" (visible moisture damages were small, area under 0,5 m^2, or none, no mould smell and no visible mould growth), "+ area" (visible moisture damages were less than 2 m^2, no smell moulds and no visible growth of moulds) and "++ area" (moisture damages were extensive, over 2 m^2, smell of moulds and visible growth of moulds). The areas of moisture damage in office buildings were not classified as above, but the building engineer detected moisture problems in all office buildings.

Analysis of micro-organisms

Air samples (n=48) were collected during January and February 1997. Air samples (V=200-400 L) for airborne micro-organisms were collected by using an Andersen six-stage impactor. Fungi were determined on 2% malt extract agar and actinomycetes on tryptone-glucose-yeast agar. The samples were incubated at 25°C for 7 days (14 days for actinomycetes), after which the colonies were counted (positive hole correction was used in air samples). The fungi were identified morphologically to genus/species/type by using a light microscope. The concentrations of fungi and bacteria were expressed in air samples as colony forming units (cfu)/m^3.

Statistics.

The statistical significance of differences between the groups of moisture damaged areas was tested by Kruskal-Walls' one way variance analysis.

RESULTS

Concentrations of micro-organisms

The geometric mean (GM) of airborne fungi concentration in schools was 24 cfu/m^3 in rooms with small (or none) water damages, 97 cfu/m^3 in rooms with moderate water damages and 132 cfu/m^3 in rooms with severe water damages. The corresponding median values were 23, 60 and 200 cfu/m^3, respectively. The concentrations of airborne fungal spores varied from 5 to 780 cfu/m^3. The concentrations differed significantly (p=0.05) between 0-, +- and ++- areas.

The GM of viable fungal spores in indoor air was 31 cfu/m^3 in office buildings with moderate or severe water damages and the corresponding median value was 40 cfu/m^3. The concentrations of fungal spores varied between 2-360 cfu/m^3. Both the GM and median values of viable actinomycetes were 3-5 cfu/m^3 in offices and schools in rooms with moderate damage and 10 cfu/m^3 in schools in rooms with severe damage.

FUNGAL FLORA

Penicillium, non-sporing isolates and yeasts were most common airborne fungi in all areas. In addition, *Aspergillus versicolor* was frequently detected in air samples in the damaged areas (+,++) in schools and offices. In schools buildings, in the damaged areas, *Aspergillus* species, *Trichoderma, Scopulariopsis* and *Acremonium* were also found more often than in non-damaged areas. In office buildings with mould problem basidiomycetes were common fungi in indoor air. Generally, in school buildings airborne fungal flora were more abundant in damaged areas (+, ++) than in non-damaged areas (0) in the schools.

DISCUSSION

The concentrations of airborne fungi and bacteria were generally low in moisture damaged schools and offices. However, in schools the concentrations increased when approaching damaged areas. The differences in the concentrations of airborne fungi found in 0-, +- and ++-areas were statistically significant. Fungal spore concentrations under 500 cfu/m^3 in indoor air in winter are considered as normal background levels in urban or suburban homes based on the Finnish guidelines (Reponen et al. 1992). In the present study, the concentrations of fungal spores in indoor air of schools and offices (GM 132 cfu/m^3) were usually lower than the Finnish guidelines for houses. This occurred even in most severely damaged area (++) in the present study. The background level of fungal spores is high in house environments due to certain household activities (Lehtonen et al.1993). Futhermore, in office environments ventilation volumes are usually high and effective air filters are used in ventilation systems which most likely affect the airborne fungal spore concentrations. The concentrations of viable fungal spores in indoor air of moisture damaged schools and day-care centers have also earlier been quite low compared to the Finnish guidelines (Reponen et al. 1994, Koskinen et al. 1995, Meklin et al. 1996). The levels of airborne fungal spores in schools have been lower than the levels in homes also in Norway (Dotterud et al. 1995 and 1996).

In the present study, fungi regarded as indicators of moisture damage such as *Acremonium, Trichoderma* and *Aspergillus versicolor,* were detected in 29 of the 48 air samples (Hyvärinen et al. 1993, Reponen et al. 1994, Koskinen et al. 1995). Prevalence of airborne *Aspergillus* spores have been higher in damp buildings than in reference ones. *Penicillium* is a common fungus in indoor air but the high concentration of airborne *Penicillium* may also indicate unusual indoor sources (Pasanen 1992, Hyvärinen et al. 1993). In the present study, *Penicillium* was a dominant fungus in indoor air and the concentrations were higher in damaged areas (+, ++) than in non-damaged areas (0). The presence of actinomycetes suggested that there was microbial growth in building materials: the prevalence of actinomycetes was more abundant in damaged

areas (+,++) than in non-damaged areas (0). The results are in accordance with earlier studies (Nevalainen et al. 1991).

According to the present findings, the guidelines for the concentrations of fungal spores in indoor air in homes are not directly suitable for other buildings and living environments such as offices and schools.

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ALLERGENS, DIAGNOSIS, AND PATHOGENESIS

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ABSTRACT

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disease caused by the ubiquitous fungus *Aspergillus*. The predominant species associated with this disease is A. fumigatus, a thermotolerant fungus. ABPA is characterized by eosinophilia, fleeting pulmonary infiltrates, central bronchiectasis, elevated serum IgE, and *Aspergillus* specific IgG and IgE. The immunodiagnosis has improved considerably in recent years due to the availability of more purified and relevant antigens for *in vitro* and *in vivo* testing. Currently, a number of recombinant proteins and peptides produced from *A. fumigatus* are being evaluated for the diagnosis of ABPA. The pathogenetic mechanism is considered to be predominantly IgE mast cell mediated tissue injury. The cytokine response detected in ABPA is of a Th2 type as evidenced by the production of IL-4, IL-5, and very little or no IFN-γ by *Aspergillus* antigen stimulated T-lymphocytes. Studies using T-cell clones from ABPA patients stimulated with recombinant *Aspergillus* allergens and synthetic peptides further confirm the characteristic Th2 response. Animal models of ABPA developed in wild type and gene knockout mice indicate a more precise mechanism of lung injury in *Aspergillus* antigen exposed animals. IL-4 knockout mice invariably showed a predominant Th1 response as against a Th2 type response in wild mice. IgE knockout mice exposed to *A. fumigatus* antigens showed airway response similar to wild type mice indicating a lesser role for IgE. Similarly, B cell deficient mice (μ -/-) failed to show any antibody response, while both wild and B cell deficient mice exposed to *Aspergillus* demonstrated identical lung pathology and airway hyperreactivity. RAG negative mice exposed to *Aspergillus* antigen failed to show airway response, although transfer of sensitized T-cells from wild type mice induced airway hyperreactivity in these animals. These results indicate that T-cells and other factors such as chemokines play a major role in the pathogenesis of ABPA, and understanding this multifactorial mechanism may aid in the diagnosis and treatment of ABPA and related diseases.

INTRODUCTION

Aspergillus fumigatus, a ubiquitous fungus, has been associated with a number of diseases in humans. These include saprophytic colonization, allergic manifestations, and invasive aspergillosis. The allergic diseases caused by *A. fumigatus* and rarely by other *Aspergillus* species are IgE mediated asthma, allergic sinusitis, allergic rhinitis, and allergic bronchopulmonary aspergillosis (Kurup VP, Kumar A. 1991;Patterson R, Greenberger PA, Roberts ML. eds. 1995;

Kurup VP, Apter AJ. eds. 1998). Allergic bronchopulmonary aspergillosis (ABPA) originally thought to be a rarity is now recognized with increasing frequency in patients with asthma and cystic fibrosis. *A. fumigatus* is a ubiquitous fungus grows at temperatures ranging from 15 to 55°C, and tolerates all climactic conditions. The spores are small and capable of penetrating the lower respiratory tract when *A. fumigatus* contaminated air is inhaled.

ABPA presents as asthma, fever, chest pain, and productive cough with blood-streaked sputum, bronchial wall thickening, and transient pulmonary infiltrates (Greenberger PA, Patterson R. 1986). The disease may progress into irreversible lung damages if not diagnosed and treated early. ABPA has been classified into five stages, which include acute, remission, exacerbation, corticosteroid dependent stage, and end stage of pulmonary fibrosis (Patterson R, Greenberger PA, Roberts ML. eds. 1995; Kurup VP, Apter AJ. eds. 1998; Patterson R, Roberts M. 1995).

ABPA has been described as an immunological disease with diagnostic characteristics of immediate and late cutaneous reactivity with *Aspergillus* antigens, elevated serum IgE and precipitating antibodies to *A. fumigatus* (Kurup VP, Apter AJ. eds. 1998; Greenberger PA, Patterson R. 1986). Central bronchiectasis and sputum and blood eosinophilia are characteristic of ABPA. Pulmonary function testing may be of value in the diagnosis of asthma and can help monitor patients with ABPA who may have excessive loss of pulmonary function parameters such as FEV_1. In-patients with progressive disease, there is persistent obstruction and reduction in airflow. The chest x-rays of patients with ABPA may demonstrate transient infiltrates resulting from mucoid impactions and proximal bronchiectasis (Kurup VP, Apter AJ. eds. 1998; Neeld DA, et al. 1990). This may progress to advance disease characterized by pulmonary fibrosis, bullae, and pneumothorax. Pulmonary pathology is variable and the lesions may have evidence of mucoid impaction, pulmonary fibrosis, eosinophilic pneumonia, granuloma, and obliterative bronchitis. However, all these features may not be present in all the patients at all time.

Laboratory Diagnosis

Demonstration of circulating antibodies against *A. fumigatus* in the sera of patients is a valuable criterion in the diagnosis. The common methods are immunodiffusion test, ELISA, and Western blots (Kurup VP, Kumar A. 1991; Kurup VP, Apter AJ. eds. 1998). Although these tests show diverse sensitivity and specificity, when properly tested and interpreted they are of value in the diagnosis. Antigen induced peripheral blood lymphocyte proliferation has proven value in diagnosis as well as understanding the immunopathogenesis of ABPA (Kurup VP, Apter AJ. eds. 1998).

The reliability of laboratory diagnosis depends on availability of reproducible reagents particularly *A. fumigatus* antigens. Antigen extracts from the whole mycelium or metabolic products secreted into the culture medium during the growth of *A. fumigatus* have been used for the detection of antibodies in the sera of patients. In recent years semi-purified antigens have been prepared which showed consistent reactivity with ABPA sera (Kurup VP, Apter AJ. eds. 1998). However standardized and well-characterized antigens are still not available for dependable use in the immunodiagnosis of ABPA and other *Aspergillus* induced diseases (Kurup VP, Kumar A. 1991; Kurup VP, Apter AJ. eds. 1998; Kurup VP, Fink JN. 1997). Although several immunoassays have been evaluated, by far, agar gel double diffusion and enzyme linked immunosorbent assays are the most frequently used diagnostic test (Kurup VP, Kumar A. 1991). With the

advent of pure and reliable antigens, automated antibody assays and cell-mediated immune response assays have started attracting recognition.

ANTIGENS OF *A. FUMIGATUS*

Improved reliability and sensitivity of immunodiagnosis have been reported with fractionated or purified antigens obtained by conventional chromatographic and electrophoretic methods, monoclonal antibody dependent purification and molecular cloning (Kurup VP, Kumar A. 1991; Kurup VP, Apter AJ. eds. 1998). Both culture filtrate and mycelial antigens from *A. fumigatus* have been characterized using methods such as polyacrylamide gel electrophoresis, isoelectric focusing, two-dimensional gel electrophoresis, and affinity chromatography. Some of the diagnostically relevant enzymes, carbohydrates, glycoproteins, and proteins from *A. fumigatus* have been purified and characterized using various purification techniques such as gel filtration, ion exchange chromatography, preparative isoelectric focusing, hydrophobic interaction chromatography and affinity purification (Kurup VP, Kumar A. 1991; Kurup VP, Apter AJ. eds 1998). However, none of these methods provided sufficient quantities of purified relevant antigens. Subsequently, hybridoma technology and molecular cloning procedures have been introduced to purify relevant *Aspergillus* allergens. Although, several major allergens from *A. fumigatus* have been purified and characterized using these methods, still such purified reagents are not available commercially for diagnostic use.

RECOMBINANT ALLERGENS OF *A. FUMIGATUS*

To circumvent the problems confronting standardization of allergenic and antigenic preparations and for the better understanding of pathogenesis and immunodiagnosis, biologically and immunologically active recombinant allergens, antigens and enzymes of *A. fumigatus* have been produced. The genes encoding a number of proteins of *A. fumigatus* have been cloned, characterized and expressed in prokaryotic systems (Banerjee B et al. 1996; Kumar A et al. 1993; Moser M et al. 1992; Moser M et al. 1994).

A 686-bp long *A. fumigatus* cDNA encoding 18 kD ribotoxin of *A. fumigatus* was cloned and expressed in Escherichia coli as a fusion protein (Moser M et al. 1992). This protein, Asp f I/a showed reactivity with IgE antibody in the sera from individuals allergic to *A. fumigatus* by ELISA, dot blots and Western blots (Moser M et al. 1994). Recombinant Asp f 1/a also elicited positive immediate type I skin reactions in individuals allergic to *A. fumigatus* but not in healthy controls, and therefore, have potential diagnostic use in *A. fumigatus* - associated diseases. Patients with ABPA showed significantly elevated levels of r Asp f I/a specific IgG4 and IgG1 but no significant rAsp f I/a - specific serum IgA levels when compared with the healthy controls. The correlation between rAsp f I/a skin test results and serologic data demonstrate potential use for recombinant allergens in diagnosis (Kurup VP, Apter AJ. eds. 1998).

A clone from the complementary (cDNA) library was found to express a 47 kD protein of *A. fumigatus* (Kurup VP, Apter AJ. eds. 1998; Kumar A et al. 1993). The recombinant protein Asp f 12 reacted with IgE and IgG class of antibodies in the sera of patients with ABPA. The deduced amino acid sequence of the partially sequenced cDNA of the clone is homologous with the heat shock protein 90 family. Another *A. fumigatus* gene encoding an allergen of 34 kD was cloned and expressed in *E. coli* (Kurup VP, Apter AJ. eds. 1998; Banerjee B et al. 1998).

The 987 bp long cDNA clone expressed a recombinant allergen Asp f 2 (Banerjee B et al. 1998). This protein exhibited binding to IgE in the serum samples of ABPA, with more patients having specific binding with central bronchiectasis. Recently a phage display library has been used to express specific proteins from *A. fumigatus*. The expression products have been displayed on the surface of filamentous phage M13 and screened for gene products binding to human serum IgE (Hemmann S et al. 1998; Crameri R. 1998). A peroxisomal protein, Asp f 3, a major allergen reacted with IgE from *Aspergillus* skin test positive asthmatics with and without ABPA (Hemmann S et al. 1998). The non-secreted *A. fumigatus* proteins, Asp f 4 and Asp f 6, showed strong binding to IgE from ABPA with very little reactivity with sera from skin test positive asthmatics without ABPA (Hemmann S et al. 1998). Thus, it can be concluded that Asp f 2, f 4, and f 6 was discriminative to ABPA while reactivity with Asp f 1 and Asp f 3 indicates sensitization. A number of *A. fumigatus* genes with diverse enzymatic activities have been cloned and sequenced from cDNA expression library on filamentous phage surface (Kurup VP, Apter AJ. eds 1998). Other allergens, rAsp f 5, rAsp f 7, rAsp f 9, and rAsp f 10 were also cloned and characterized from phage display library of *A. fumigatus* cDNA, also exhibited binding to IgE from the sera of ABPA patients (Crameri R. 1998).

Immunopathogenesis of ABPA

The immune response in ABPA is assumed to be $CD4^+$ T-helper 2 (Th_2) cells mediated (Kurup VP, Apter AJ. eds. 1998; Knutsen AP, Chauhan B, Slavin RG. 1998). The Th_2 response is manifested by the production of IL-4, IL-5, IL-10, and IL-13 by *Af* stimulated T-lymphocytes (Kurup VP, Apter AJ. eds. 1998; Knutsen AP, Chauhan B, Slavin RG. 1998; Kurup VP et al. 1998). IL-4 and IL-13 induce IgE switching of B-cells, while Il-5 induces eosinophil recruitment and differentiation. The current evidence although incomplete suggests the presence of a complex immunopathogensis in ABPA. The T-cell lympho-proliferative response to both crude and purified *Aspergillus* antigens and T and B cell interactions in ABPA have been studied (Knutsen AP, Chauhan B, Slavin RG. 1998). Antigen stimulated T-cells from ABPA patients enhanced the B-cell IgE synthesis. T-cell lines specific to Asp f 1produced Th_2 cytokines *in vitro* when stimulated with the antigen. The T-cell clones generated from ABPA patients against *A. fumigatus* antigens were either Th_2 or Th_0 cells. The limited study using T-cell clones from ABPA patients also suggests a predominant Th_2 response but a number of factors determine the Th_1 and Th_2 cross regulation and the cytokine-chemokine participation in the immunopathogenesis of ABPA (Knutsen AP, Chauhan B, Slavin RG. 1998; Kurup VP et al. 1998).

Our understanding on the immunoregulation is mostly derived from animal models of ABPA (Grunig G et al. 1998). Mice exposed to *A. fumigatus* antigens developed elevated levels of IgE in the serum and eosinophilia in the peripheral blood, lung, and bone marrow. The high IgE production was attributed to the expression of IL-4 by activated $CD4^+$ Th_2 lymphocytes. Anti-IL-4 treatment down-regulated serum IgG_1 and IgE, but enhanced IgG_{2a} in these models. This was further confirmed in IL-4 knockout mice where a predominantly Th_1 response was demonstrable (Kurup VP et al. 1998). However, lung injury observed in IL-4 knockout mice were similar to those observed in wild mice challenged with *A. fumigatus* antigen. As no IgE and IL-4 are demonstrable in these IL-4 knockout animals, the response may be due to the infiltration of eosinophils into the airways due to local production of cytokines and chemokines capable of attracting and stimulating eosinophils. Anti-IL-5 injection resulted in the abrogation of all the

eosinophils, while anti-IFN-γ treatment led to a Th2 response in the exposed animals with enhanced serum IgE and markedly increased pulmonary and peripheral blood eosinophils (Kurup VP et al. 1998; Grunig G et al. 1998).

In all murine models of allergic aspergillosis, it was found that IL-4 is critical for the production of IgE. However, IgE knockout mice exposed to *A. fumigatus* antigens demonstrated pulmonary infiltration of eosinophils and airway resistance. Similarly in mice with targeted mδ-chain deficiency with no mature B-cells or antibody production, demonstrated pathologic and physiological responses similar to wild animals exposed to *A. fumigatus* antigen. These findings indicate that B-cells and antibodies have very little role in ABPA (Kurup VP et al. 1998). Contrary to this, our studies with IL-4 knockout mice indicate no airway resistance in *A. fumigatus* antigen exposed animals in spite of the similarity of pulmonary inflammatory changes detected in wild mice exposed to *A. fumigatus*. However, recombinase-activating gene deficient mice (RAG -/-) failed to develop significant allergic response when exposed to *A. fumigatus*. In these mice, sensitized $CD4^+$ T-cells alone was sufficient for restoring allergen induced airway hyperreactivity and allergic inflammation. Thus it can be concluded that ABPA is a T-cell mediated condition where $CD4^+$ T-cell play a major role in the pathogenesis, although the role of IgE and eosinophils needs further elucidation (Knutsen AP, Chauhan B, Slavin RG. 1998; Kurup VP et al. 1998).

The currently available information indicates that ABPA is a Th2 mediated disease. A marked increase in the Th2 response both systemically and in the lungs can be demonstrable in experimental animals. In some ABPA patients and in the models a clear-cut Th1/Th2 response may not be discernible. This may perhaps be due to the nature of the antigens, dose, antigen presentation and/or due to the underlying host factors. Further studies are needed to understand the role of cytokines, chemokines, and other factors in the pathogenesis of ABPA. Such informations are important in diagnosis, patient care including treatment, immunotherapy, and in developing a suitable vaccine. However, further studies are essential to confirm these results.

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UP-DATE

EXPOSURE ASSESSMENT AND HEALTH EFFECTS

- A FOLLOW-UP MEETING TO THE 3rd INTERNATIONAL CONFERENCE ON FUNGI, MYCOTOXINS AND BIOAEROSOLS (Saratoga Springs, N.Y.) *

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BACKGROUND

The purpose of the meeting was to update the present state of knowledge and understanding of the microbial problems due to damp and moisture problems in buildings. As the complex issues of exposure assessment, health effects. building diagnostics, refurbishment and remediation are major areas of concern among the indoor practitioners, the follow-up meeting was an important satellite event of Healthy Building 2000 in Espoo, Finland, August 2000.

AIM AND SCOPE

The aim of the follow-up meeting was to provide a concise summary of what was achieved in the expert Conference in Saratoga Springs 1998 and about the content of the 650-pages Proceedings book (Johanning, ed. 1999). The present and future practices, policies and focuses of the research were also discussed.

STATE OF THE ART

At present, it is evident that we are dealing with a phenomenon which obviously is a public health concern but which also can be regarded as indoor air quality issue, an issue of building design and construction, building maintenance and building economy. It is a question of a complex process starting from moisture damages or other failure to control the moisture conditions in a building, followed by development of microbial growth causing exposure to harmful agents to the occupants and development of many health effects, some of which are not well understood. In some rare cases, the health consequences maybe be very serious but most commonly, they are conditions of reversible nature. The emphasis should be on the early detection and prevention of the problems.

The variety of indoor air complaints associated with moisture damage include unpleasant odors, emission of chemicals from the construction materials (e.g. volatile organic compounds, VOC), organic and inorganic dusts, and mites together with excess temperatures and insuffi-

* See final document

cient ventilation. Microbes produce spores and various kinds of mycotoxins and bacterial toxins, bacterial endotoxins, beta-glucans, and microbial volatile organic compounds (MVOC) that may all have adverse effects to human health. In the clinical setting the studied health effects may be the cumulative result of such exposures. Innovative exposure assessments and medical evaluations are trying to address this complex problem.

Exposure assessment - sampling and analytical methods

Three introductions were given in the field of exposure assessment: the sampling methods of bioaerosols, interpretation of results and analytical aspects for mycotoxins were shortly reviewed. Most components and products of microbial origin occur in air as particles, and thus an essential element of bioaerosol collection is the efficiency of sampling, including a physical and biological component. Overall sampling efficiency is usually a trade-off between those two components: if the physical collection efficiency is high, the sampling is causing injury for the collected biological particles, decreasing the biological efficiency of the sampling. Among the present methods of microbial sampling, those based on culturing only detect the viable microorganisms.

As the majority of airborne microbes are probably not culturable in the given conditions, and traditional aerosol sampling techniques add to physical stress for the microbial cells, it is evident that those methods severely underestimate the actual biological load in the air.

Physical and biological efficiencies of various sampling devices have been assessed. In impingers, water evaporates rapidly, decreasing the sampling efficiency, but viscous fluids (e.g., glycerol and mineral oil) do not have this drawback. A new sample based on centrifugal impingement, has been developed to combine the advantages of existing bioaerosol samplers and overcome their limitations. It has high physical collection efficiency but still is a gentle method to collect culturable bioaerosol particles. Furthermore, it can be used for long-time sampling of biological aerosols. If other than culturable methods are used for analysis, filter sampling is one choice for collection method. A new filter sampler has been developed which seems to be a promising tool to effectively collect biological particles of wide size range.

Detection of the sampled microbes is mainly based on viable microbes, but other methods were listed as well. PCR techniques which allow specific detection of certain microbes independent of the viability of the organism, are being developed for indoor sampling purposes. Measurements of ergosterol, glucans and endotoxins provide an assessment of total load of microbial material or its fraction. Methods focusing on the biological activity of the collected material include cytotoxicity tests in a cell culture and determination or haemolytic activity or siderophore production, which are methods used traditionally in bacteriology but now applied into environmental sampling.

Mycotoxin analytics is complicated because of the wide range of the chemical nature of the toxins and the large biological variation in their production. In order to quantify the toxins, some information is needed about what organisms are involved and thus, what toxins are expected in the sample. The need to develop high-efficiency sampling of mycotoxins, which occur in very low concentrations in air, was discussed. Another way to determine toxins is to assess the toxicity of the sample with various in vitro toxicity tests.

As a conclusion of the methods available for exposure assessment, they are still under development along with the accumulating evidence about the causal relationships between the health effects and different agents. Most methods of exposure assessment need still more validation in relation to the observed health effects.

Health effects

The health effects of indoor air microbes, molds and bacteria can be divided into following categories:

Irritative and Nonspecific Symptom

Many non-specific temporal complaints have been described by various investigators of health effects in association with indoor moisture and mold exposure, These include: irritation of the skin and mucous membrane, eye or nasal itching and dryness, prolonged sore throat, hoarseness, dry cough, chest discomfort, as well as nausea, vomiting, headaches, back aches, joint pain, and low grade fever. Usually those complaints subside rapidly with removal from the exposure. Effects may be related to irritant-toxic effects of bacteria and fungi causing localized and non-specific inflammatory effects. Gram-positive bacteria, beta-glucans and endotoxins may be related to such inflammatory effects based on in-vitro and in-vivo studies.

Infections

Fungi found in indoor environments are not typically causing primary infections, but may lower the body resistance and facilitate secondary type infections. In increased frequency of common respiratory infections and sometimes intestinal infections are reported by people with significant allergenic and toxigenic fungal exposure. This has been also described in epidemiological studies of children and adults residing in moldy buildings for prolonged time. In rare cases aspergillosis of the bronchial, nasal sinuses and vertebra has been linked to indoor exposure. Invasive fungal diseases secondary to fungal exposure appear rare in immune-competent. healthy individuals.

Allergic Diseases

Several fungi are known allergens causing type-I allergic rhinitis, asthma and allergic conjunctivitis. Alternaria, Cladosporium, Aspergillus, and Botrytis commonly occurring in outdoor air are allergenic. Allergenic molds that commonly occur in indoor air are e.g. Penicillium, Aspergillus, Mucor and Rhizopus. Other important allergens are Fusarium and Aureobasidium common in farming environment and in wood materials. It is impossible to give any complete list of allergenic microbes because new species are continuously detected and reported. Only a few fungal allergens have been precisely characterized. IgE-mediated allergy to molds and other fungi can be proved by skin prick tests or by detection of antigen-specific IgE-antibodies in a serum sample with any of the available test kits (RAST, Phadiatop, CLA-Mast etc.).The

estimates of mold allergy prevalence vary in different populations (5-10%). In selected populations like asthmatics in humid climate prevalence of mold sensitivity may much higher.

In population studies, both increased risk of asthma and asthmatic symptoms have been found in association of mold exposure or damp housing. The association of the exposure with asthma, wheezing and other symptoms of asthma has been shown in several countries and climate.

Alveolitis and Organic Dust Toxic Syndrome

The most severe pulmonary disease caused by molds and actinomycetes is allergic alveolitis (hypersensitivity pneumonitis). Alveolitis is caused by any biological dust that has particles smaller than 10 micrometers. Some fungi, i.e. Acremonium, Penicillium, Aspergillus and additionally actinomycetes have spores that are small enough to reach the alveoli. Alveolitis is cell-mediated type IV allergic reaction which leads to lymphocyte cell inflammation and tissue damage in lungs and may lead to pulmonary fibrosis in long term exposure. Allergic alveolitis has traditionally been reported in occupational exposure situations and seldom in indoor air exposure. The differential diagnosis between alveolitis and organic dust toxic syndrome (ODTS) is difficult in the clinical setting. Symptoms of ODTS seem to occur relatively often in mold exposure both in work places and in apartments. Both ODTS and alveolar inflammation maybe under diagnosed in indoor air exposure.

Chronic Bronchitis

The association between microbial exposure and high prevalence of chronic bronchitis has been reported among non-smoking farmers who have mold exposure related to their work. Similar preliminary results has been found between indoor air exposure and symptoms of chronic bronchitis. The increased risk of chronic bronchitis associated with living in water-damaged buildings was statistically significant when adjusted for age, sex, atopy and smoking. Chronic bronchitis occurs rarely in children and the prevalence increases in older age groups. The possible association between molds in indoor air and symptoms of chronic bronchitis needs further attention in future research.

Health Effects of Mycotoxins

Mycotoxin related adverse health effects are gaining more attention, since many of the observed clinical findings in patients with a known history of significant and proven toxic fungal exposure cannot be explained with allergy or simple irritant exposure pathology. Typical patient complaints are recurrent colds and flu-like illness, general malaise, sore throat, headache, fatigue, diarrhea, dermatitis and irritation of skin, as well as fatigue and impaired immune function and additionally neuromuscular disturbances, rhinitis and pneumonia.

Mycotoxins are toxic secondary metabolites of the fungi. Spores and mycelial fragments carry the toxins or they may be absorbed on dust particles. Mycotoxins are absorbed to man or animals via airways or through the skin because most of them are lipid soluble. Mycotoxins are produced by various species of fungi, such as Aspergillus versicolor, Fusarium, Trichoderma,

Cephalosporium, Chaetomium and Stachybotrys. Immunogenic effects have been shown in animal and human studies associated with specific mycotoxins. For example, trichothecenes inhibit protein synthesis and damage DNA. Immune suppression may lead to secondary bacterial, viral and parasitic infections. Recently hemorrhagic hemosiderosis have been detected in small children living in homes with widespread Stachybotrys and other mold infestation. More detailed information of mycotoxin exposure routes and mechanisms of health effects is needed.

Other Health Effects

Other adverse health effects either suspected or described in association with mold exposure are cancer, disturbances of reproductive health and neurotoxicity. Aflatoxin is classified to be carcinogenic in humans. Several other toxins are carcinogens in animals when animal feed is contaminated. There is epidemiological evidence of possible carcinogenic effects of inhaled molds based on few occupational studies related to intense exposure situations. Only very preliminary work has been done to assess the association of mold exposure and reproductive outcomes.

Some mycotoxins may cause neurological symptoms such as tremor, muscle weakness, extreme fatigue and apathy. All these symptoms have been described in humans and in anirnals. More studies are needed to further investigate this observed clinical findings and statistical associations. Improved environmental exposure assessment and characterization is currently being developed.

FURTHER RESEARCH NEED AND TOPICS FOR FURTHER DISCUSSION - A FUTURE MEETING!

The participants of the Saratoga Springs Bioaerosols, fungi and mycotoxins - follow up meeting agreed after a discussion, that a future meeting would be highly beneficial to discuss the following points among an inter-disciplinary group of researchers and practitioners:

A. Exposure assessment

1. Do we need more or less sampling methods?
2. Mycology versus and toxicology assessment
3. Consequences for the "real-world"
4. Laboratory standardization
5. Resources, Cost and practicality
6. Regional differences and priorities

B. Health, Medical issues

1. Diagnostic criteria and tools
2. Treatment options
3. Neurological effects, Endocrinology, Rheumatology, Reproductive health

C. Public health, Prevention.

1. Education of physical, public, technical expertise exchange

2. Prevention: architecture, building maintenance
3. Guidelines (harmonization of standards)?
4. Threshold levels?
5. Indicators for practical measures

Others

D. What happens to buildings and fungi over time?

E. What mycotoxins are important?

1. Improved and innovative sampling methods
2. Analytical methods

F. Which populations are at increased risk and what should be done about them?

1. Children, hospital elder, pregnant women
2. Women with endocrinology changes by Hx

G. What experimental work is necessary?

H. Prevalence of moisture and fungal problems?

I. Cost- Risk assessment

J. Team work approaches - practical experience - "a common language"

K. Progress and feed back control

1. Methods
2. Devices
3. Cooperative work

L. Public health policy implication

1. Recommendation to health departments and governmental agencies

M. Education of colleagues and professionals

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APPENDIX:

Epidemiology of symptoms/disorders in moisture problem buildings

Country	Age	N	Symptoms/disorders	OR *)	Reference
Scotland	7 yrs	1000	bronchial wheezing	3.70(2.22-6.15)	Strachan1988
England Scotland	NI	1169	bronchial wheezing	1.9	Platt et at. 1989
Sweden	NI	5301	chronic cough	2.3	Andrae et al.1988
			exercise induced cough	1.7	
			allergic asthma	1.8	
Netherland	8-12yrs	4600	bronchial wheezing	1.79(1.44-2.32)	Brunekreff et al.1989
			cough	2.12(1.64-2.73)	
			bronchitis	1.48(1.17-1.87)	
			asthma	1.27(0.93-1.74)	
Canada	5-8 yrs	15 000	asthma	1.46 (1.22-1.74)	Dekker et al. 1991
			bronchial wheezing	1.61 (1.39-1.85)	
Canada	5-8 yrs	13 495	bronchitis	1.32(1.06-1.39)	Dales et al. 1991
			cough	1.89(1.58-2.26)	

*) OR=odds ratio (95% confidence interval), NI= no information

GUIDELINES ON ASSESSMENT AND REMEDIATION OF FUNGI IN INDOOR ENVIRONMENTS (2000)

NEW YORK CITY DEPARTMENT OF HEALTH BUREAU OF ENVIRONMENTAL & OCCUPATIONAL DISEASE EPIDEMIOLOGY

EXECUTIVE SUMMARY

On May 7, 1993, the New York City Department of Health (DOH), the New York City Human Resources Administration (HRA), and the Mt. Sinai Occupational Health Clinic convened an expert panel on *Stachybotrys atra* in Indoor Environments. The purpose of the panel was to develop policies for medical and environmental evaluation and intervention to address *Stachybotrys atra* (now known as *Stachybotrys chartarum* (SC)) contamination. The original guidelines were developed because of mold growth problems in several New York City buildings in the early 1990's. This document revises and expands the original guidelines to include all fungi (mold). It is based both on a review of the literature regarding fungi and on comments obtained by a review panel consisting of experts in the fields of microbiology and health sciences. It is intended for use by building engineers and management, but is available for general distribution to anyone concerned about fungal contamination, such as environmental consultants, health professionals, or the general public.

We are expanding the guidelines to be inclusive of all fungi for several reasons:

- Many fungi (e.g., species of *Aspergillus, Penicillium, Fusarium, Trichoderma,* and *Memnoniella*) in addition to SC can produce potent mycotoxins, some of which are identical to compounds produced by SC. Mycotoxins are fungal metabolites that have been identified as toxic agents. For this reason, SC cannot be treated as uniquely toxic in indoor environments.
- People performing renovations/cleaning of widespread fungal contamination may be at risk for developing Organic Dust Toxic Syndrome (ODTS) or Hypersensitivity Pneumonitis (HP). ODTS may occur after a *single heavy* exposure to dust contaminated with fungi and produces flu-like symptoms. It differs from HP in that it is not an immune-mediated disease and does not require repeated exposures to the same causative agent. A variety of biological agents may cause OTDS including common species of fungi. HP may occur after repeated exposures to an allergen and can result in permanent lung damage.
- Fungi can cause allergic reactions. The most common symptoms are runny nose, eye irritation, cough, congestion, and aggravation of asthma.

Fungi are present almost everywhere in indoor and outdoor environments. The most common symptoms of fungal exposure are runny nose, eye irritation, cough, congestion, and aggravation of asthma. Although there is evidence documenting severe health effects of fungi in humans, most of this evidence is derived from ingestion of contaminated foods (i.e., grain and peanut products) or occupational exposures in agricultural settings where inhalation exposures were very high. With the possible exception of remediation to very heavily contaminated

indoor environments, such high-level exposures are not expected to occur while performing remedial work.

There have been reports linking health effects in office workers to offices contaminated with moldy surfaces and in residents of homes contaminated with fungal growth. Symptoms, such as fatigue, respiratory ailments, and eye irritation were typically observed in these cases. Some studies have suggested an association between SC and pulmonary hemorrhage/hemosiderosis in infants, generally those less than six months old. Pulmonary hemosiderosis is an uncommon condition that results from bleeding in the lungs. The cause of this condition is unknown, but may result from a combination of environmental contaminants and conditions (e.g., smoking, fungal contaminants and other bioaerosols, and water-damaged homes), and currently its association with SC is unproven.

The focus of this guidance document addresses mold contamination of building components (walls, ventilation systems, support beams, etc.) that are chronically moist or water damaged. Occupants should address common household sources of mold, such as mold found in bathroom tubs or between tiles with household cleaners. Moldy food (e.g., breads, fruits, etc.) should be discarded.

Building materials supporting fungal growth must be remediated as *rapidly as possible* in order to ensure a healthy environment. Repair of the defects that led to water accumulation (or elevated humidity) should be conducted in conjunction with or prior to fungal remediation. Specific methods of assessing and remediating fungal contamination should be based on the extent of visible contamination and underlying damage. The simplest and most expedient remediation that is reasonable, and properly and safely removes fungal contamination, should be used. Remediation and assessment methods are described in this document.

The use of respiratory protection, gloves, and eye protection is recommended. Extensive contamination, particularly if heating, ventilating, air conditioning (HVAC) systems or large occupied spaces are involved, should be assessed by an experienced health and safety professional and remediated by personnel with training and experience handling environmentally contaminated materials. Lesser areas of contamination can usually be assessed and remediated by building maintenance personnel. In order to prevent contamination from recurring, underlying defects causing moisture buildup and water damage must be addressed. Effective communication with building occupants is an essential component of all remedial efforts.

Fungi in buildings may cause or exacerbate symptoms of allergies (such as wheezing, chest tightness, shortness of breath, nasal congestion, and eye irritation), especially in persons who have a history of allergic diseases (such as asthma and rhinitis). Individuals with persistent health problems that appear to be related to fungi or other bioaerosol exposure should see their physicians for a referral to practitioners who are trained in occupational/environmental medicine or related specialties and are knowledgeable about these types of exposures. Decisions about removing individuals from an affected area must be based on the results of such medical evaluation, and be made on a case-by-case basis. Except in cases of widespread fungal contamination that are linked to illnesses throughout a building, building-wide evacuation is not indicated.

In summary, prompt remediation of contaminated material and infrastructure repair is the primary response to fungal contamination in buildings. Emphasis should be placed on preventing

contamination through proper building and HVAC system maintenance and prompt repair of water damage.

This document is not a legal mandate and should be used as a guideline. Currently there are no United States Federal, New York State, or New York City regulations for evaluating potential health effects of fungal contamination and remediation. These guidelines are subject to change as more information regarding fungal contaminants becomes available.

INTRODUCTION

On May 7, 1993, the New York City Department of Health (DOH), the New York City Human Resources Administration (HRA), and the Mt. Sinai Occupational Health Clinic convened an expert panel on *Stachybotrys atra* in Indoor Environments. The purpose of the panel was to develop policies for medical and environmental evaluation and intervention to address *Stachybotrys atra* (now known as *Stachybotrys chartarum* (SC)) contamination. The original guidelines were developed because of mold growth problems in several New York City buildings in the early 1990's. This document revises and expands the original guidelines to include all fungi (mold). It is based both on a review of the literature regarding fungi and on comments obtained by a review panel consisting of experts in the fields of microbiology and health sciences. It is intended for use by building engineers and management, but is available for general distribution to anyone concerned about fungal contamination, such as environmental consultants, health professionals, or the general public.

This document contains a discussion of potential health effects; medical evaluations; environmental assessments; protocols for remediation; and a discussion of risk communication strategy. The guidelines are divided into four sections:

1. Health Issues; 2. Environmental Assessment; 3. Remediation; and 4. Hazard Communication.

We are expanding the guidelines to be inclusive of all fungi for several reasons:

- Many fungi (e.g., species of *Aspergillus, Penicillium, Fusarium, Trichoderma*, and *Memnoniella*) in addition to SC can produce potent mycotoxins, some of which are identical to compounds produced by SC. (Bata A, et al. 1985; Jarvis B, 1990; Yang C, Johanning E, 1996; Jarvis B, Mazzola E. 1982.) Mycotoxins are fungal metabolites that have been identified as toxic agents. For this reason, SC cannot be treated as uniquely toxic in indoor environments.
- People performing renovations/cleaning of widespread fungal contamination may be at risk for developing Organic Dust Toxic Syndrome (ODTS) or Hypersensitivity Pneumonitis (HP). ODTS may occur after a single heavy exposure to dust contaminated with fungi and produces flu-like symptoms. It differs from HP in that it is not an immune-mediated disease and does not require repeated exposures to the same causative agent. A variety of biological agents may cause OTDS including common species of fungi. HP may occur after repeated exposures to an allergen and can result in permanent lung damage. (Von Essen S, et al. 1990; Richerson H. 1990 , Malmberg P, et al. 1990; Malmberg P. 1990; Yoshida K, Masayuki A, Shukuro A. 1989; Lecours R, Laviolette M, Cormier Y. 1986.)

- Fungi can cause allergic reactions. The most common symptoms are runny nose, eye irritation, cough, congestion, and aggravation of asthma. (Levetin E. 1995; Husman T. 1996.)

Fungi are present almost everywhere in indoor and outdoor environments. The most common symptoms of fungal exposure are runny nose, eye irritation, cough, congestion, and aggravation of asthma. Although there is evidence documenting severe health effects of fungi in humans, most of this evidence is derived from ingestion of contaminated foods (i.e., grain and peanut products) or occupational exposures in agricultural settings where inhalation exposures were very high. (Miller J D. 1995; Cookingham C, Solomon W. 1995.). With the possible exception of remediation to very heavily contaminated indoor environments, such high level exposures are not expected to occur while performing remedial work. (Rautiala S, et al. 1998.)

There have been reports linking health effects in office workers to offices contaminated with moldy surfaces and in residents of homes contaminated with fungal growth. (Husman T. 1996; Dales R, et al. 1991; Hodgson M, et al. 1998; Croft W, Jarvis B, Yatawara C. 1986; DeKoster J, Thorne P. 1995; Johanning E, et al. 1996.) Symptoms, such as fatigue, respiratory ailments, and eye irritation were typically observed in these cases.

Some studies have suggested an association between SC and pulmonary hemorrhage/hemosiderosis in infants, generally those less than six months old. Pulmonary hemosiderosis is an uncommon condition that results from bleeding in the lungs. The cause of this condition is unknown, but may result from a combination of environmental contaminants and conditions (e.g., smoking, other microbial contaminants, and water-damaged homes), and currently its association with SC is unproven. (Montana E, et al. 1997; Etzel R, et al. 1998; CDC. Update: 2000.)

The focus of this guidance document addresses mold contamination of building components (walls, ventilation systems, support beams, etc.) that are chronically moist or water damaged. Occupants should address common household sources of mold, such as mold found in bathroom tubs or between tiles with household cleaners. Moldy food (e.g., breads, fruits, etc.) should be discarded.

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1. HEALTH ISSUES

1.1 Health Effects

Inhalation of fungal spores, fragments (parts), or metabolites (e.g., mycotoxins and volatile organic compounds) from a wide variety of fungi may lead to or exacerbate immunologic (allergic) reactions, cause toxic effects, or cause infections. (Levetin E. 1995: Husman T. 1996; Burge H, Otten J. 1999.)

There are only a limited number of documented cases of health problems from indoor exposure to fungi. The intensity of exposure and health effects seen in studies of fungal exposure in the indoor environment was typically much less severe than those that were experienced by

agricultural workers but were of a long-term duration.(Von Essen S, et al. 1990; ,Richerson H. 1990: Malmberg P, et al. 1990; Malmberg P. 1990; Yoshida K, Masayuki A, Shukuro A. 1989; Lecours R, Laviolette M, Cormier Y. 1986; Husman T. 1996; Cookingham C, Solomon W. 1995; Dales R, et al. 1991; Hodgson M, et al. 1998; Croft W, Jarvis B, Yatawara C. 1986; DeKoster J, Thorne P. 1995; Johanning E, et al.. 1996; do Pico G. 1992; Hodgson M, et al. 1985; Weltermann B, et al.. 1998.) Illnesses can result from both high level, short-term exposures and lower level, long-term exposures. The most common symptoms reported from exposures in indoor environments are runny nose, eye irritation, cough, congestion, aggravation of asthma, headache, and fatigue. (Levetin E. 1995; Husman T. 1996; Dales R, et al.. 1991; Hodgson M, et al. 1998; Croft W, Jarvis B, Yatawara C. 1986; DeKoster J, Thorne P. 1995; Johanning E, et al. 1996.)

The presence of fungi on building materials as identified by a visual assessment or by bulk/surface sampling results does not necessitate that people will be exposed or exhibit health effects. In order for humans to be exposed indoors, fungal spores, fragments, or metabolites must be released into the air and inhaled, physically contacted (dermal exposure), or ingested. Whether or not symptoms develop in people exposed to fungi depends on the nature of the fungal material (e.g., allergenic, toxic, or infectious), the amount of exposure, and the susceptibility of exposed persons. Susceptibility varies with the genetic predisposition (e.g., allergic reactions do not always occur in all individuals), age, state of health, and concurrent exposures. For these reasons, and because measurements of exposure are not standardized and biological markers of exposure to fungi are largely unknown, it is not possible to determine "safe" or "unsafe" levels of exposure for people in general.

1.1.1 Immunological Effects

Immunological reactions include asthma, HP, and allergic rhinitis. Contact with fungi may also lead to dermatitis. It is thought that these conditions are caused by an immune response to fungal agents. The most common symptoms associated with allergic reactions are runny nose, eye irritation, cough, congestion, and aggravation of asthma. (Levetin E. 1995; Husman T. 1996.) HP may occur after repeated exposures to an allergen and can result in permanent lung damage. HP has typically been associated with repeated heavy exposures in agricultural settings but has also been reported in office settings. (do Pico G. 1992; Hodgson M, et al. 1985; Weltermann B, et al. 1998.) Exposure to fungi through renovation work may also lead to initiation or exacerbation of allergic or respiratory symptoms.

1.1.2 Toxic Effects

A wide variety of symptoms have been attributed to the toxic effects of fungi. Symptoms, such as fatigue, nausea, and headaches, and respiratory and eye irritation have been reported. Some of the symptoms related to fungal exposure are non-specific, such as discomfort, inability to concentrate, and fatigue. (Levetin E. 1995; Husman T. 1996; Dales R, et al. 1991; Hodgson M, et al. 1998; Croft W, Jarvis B, Yatawara C. 1986; DeKoster J, Thorne P. 1995; Johanning E, et al.. 1996.) Severe illnesses such as OTDS and pulmonary hemosiderosis have also been attributed to fungal exposures (Von Essen S, et al. 1990; Richerson H. 1990: Malmberg P, et al. 1990;.

Malmberg P. 1990; Yoshida K, Masayuki A, Shukuro A. 1989; Lecours R, Laviolette M, Cormier Y. 1986; Montana E, et al. 1997; Etzel R, et al. 1998.)

ODTS describes the abrupt onset of fever, flu-like symptoms, and respiratory symptoms in the hours following a single, heavy exposure to dust containing organic material including fungi. It differs from HP in that it is not an immune-mediated disease and does not require repeated exposures to the same causative agent. ODTS may be caused by a variety of biological agents including common species of fungi (e.g., species of *Aspergillus* and *Penicillium*). ODTS has been documented in farm workers handling contaminated material but is also of concern to workers performing renovation work on building materials contaminated with fungi. (Von Essen S, et al. 1990; Richerson H. 1990: Malmberg P, et al. 1990; Malmberg P. 1990; Yoshida K, Masayuki A, Shukuro A. 1989; Lecours R, Laviolette M, Cormier Y. 1986.)

Some studies have suggested an association between SC and pulmonary hemorrhage/hemosiderosis in infants, generally those less than six months old. Pulmonary hemosiderosis is an uncommon condition that results from bleeding in the lungs. The cause of this condition is unknown, but may result from a combination of environmental contaminants and conditions (e.g., smoking, fungal contaminants and other bioaerosols, and water-damaged homes), and currently its association with SC is unproven. (Montana E, et al. 1997; Etzel R, et al. 1998; CDC. Update: 2000.)

1.1.3 Infectious Disease

Only a small group of fungi have been associated with infectious disease. Aspergillosis is an infectious disease that can occur in immunosuppressed persons. Health effects in this population can be severe. Several species of *Aspergillus* are known to cause aspergillosis. The most common is *Aspergillus fumigatus*. Exposure to this common mold, even to high concentrations, is unlikely to cause infection in a healthy person. (Levetin E. 1995; Burge H, Otten J. 1999.)

Exposure to fungi associated with bird and bat droppings (e.g., *Histoplasma capsulatum* and *Cryptococcus neoformans*) can lead to health effects, usually transient flu-like illnesses, in healthy individuals. Severe health effects are primarily encountered in immunocompromised persons. (Burge H, Otten J. 1999; Band J. 1986; Bertolini R. 1988.)

1.2 Medical Evaluation

Individuals with persistent health problems that appear to be related to fungi or other bioaerosol exposure should see their physicians for a referral to practitioners who are trained in occupational/environmental medicine or related specialties and are knowledgeable about these types of exposures. Infants (less than 12 months old) who are experiencing non-traumatic nosebleeds or are residing in dwellings with damp or moldy conditions and are experiencing breathing difficulties should receive a medical evaluation to screen for alveolar hemorrhage. Following this evaluation, infants who are suspected of having alveolar hemorrhaging should be referred to a pediatric pulmonologist. Infants diagnosed with pulmonary hemosiderosis and/or pulmonary hemorrhaging should not be returned to dwellings until remediation and air testing are completed.

Clinical tests that can determine the source, place, or time of exposure to fungi or their products are not currently available. Antibodies developed by exposed persons to fungal agents can only document that exposure has occurred. Since exposure to fungi routinely occurs in both outdoor and indoor environments this information is of limited value.

1.3 Medical Relocation

Infants (less than 12 months old), persons recovering from recent surgery, or people with immune suppression, asthma, hypersensitivity pneumonitis, severe allergies, sinusitis, or other chronic inflammatory lung diseases may be at greater risk for developing health problems associated with certain fungi. Such persons should be removed from the affected area during remediation (see Section 3, Remediation). Persons diagnosed with fungal related diseases should not be returned to the affected areas until remediation and air testing are completed.

Except in cases of widespread fungal contamination that are linked to illnesses throughout a building, a building-wide evacuation is not indicated. A trained occupational/environmental health practitioner should base decisions about medical removals in the occupational setting on the results of a clinical assessment.

2. ENVIRONMENTAL ASSESSMENT

The presence of mold, water damage, or musty odors should be addressed immediately. In all instances, any source(s) of water must be stopped and the extent of water damaged determined. Water damaged materials should be dried and repaired. Mold damaged materials should be remediated in accordance with this document (see Section 3, Remediation).

2.1 Visual Inspection

A visual inspection is the most important initial step in identifying a possible contamination problem. The extent of any water damage and mold growth should be visually assessed. This assessment is important in determining remedial strategies. Ventilation systems should also be visually checked, particularly for damp filters but also for damp conditions elsewhere in the system and overall cleanliness. Ceiling tiles, gypsum wallboard (sheetrock), cardboard, paper, and other cellulosic surfaces should be given careful attention during a visual inspection. The use of equipment such as a boroscope, to view spaces in ductwork or behind walls, or a moisture meter, to detect moisture in building materials, may be helpful in identifying hidden sources of fungal growth and the extent of water damage.

2.2 Bulk/ Surface Sampling

a. Bulk or surface sampling is not required to undertake a remediation. Remediation (as described in Section 3, Remediation) of visually identified fungal contamination should proceed without further evaluation.

b. Bulk or surface samples may need to be collected to identify specific fungal contaminants as part of a medical evaluation if occupants are experiencing symptoms which may be relat-

ed to fungal exposure or to identify the presence or absence of mold if a visual inspection is equivocal (e.g., discoloration, and staining).

c. An individual trained in appropriate sampling methodology should perform bulk or surface sampling. Bulk samples are usually collected from visibly moldy surfaces by scraping or cutting materials with a clean tool into a clean plastic bag. Surface samples are usually collected by wiping a measured area with a sterile swab or by stripping the suspect surface with clear tape. Surface sampling is less destructive than bulk sampling. Other sampling methods may also be available. A laboratory specializing in mycology should be consulted for specific sampling and delivery instructions.

2.3 Air Monitoring

a. Air sampling for fungi should not be part of a routine assessment. This is because decisions about appropriate remediation strategies can usually be made on the basis of a visual inspection. In addition, air-sampling methods for some fungi are prone to false negative results and therefore cannot be used to definitively rule out contamination.

b. Air monitoring may be necessary if an individual(s) has been diagnosed with a disease that is or may be associated with a fungal exposure (e.g., pulmonary hemorrhage/hemosiderosis, and aspergillosis).

c. Air monitoring may be necessary if there is evidence from a visual inspection or bulk sampling that ventilation systems may be contaminated. The purpose of such air monitoring is to assess the extent of contamination throughout a building. It is preferable to conduct sampling while ventilation systems are operating.

d. Air monitoring may be necessary if the presence of mold is suspected (e.g., musty odors) but cannot be identified by a visual inspection or bulk sampling (e.g., mold growth behind walls). The purpose of such air monitoring is to determine the location and/or extent of contamination.

e. If air monitoring is performed, for comparative purposes, outdoor air samples should be collected concurrently at an air intake, if possible, and at a location representative of outdoor air. For additional information on air sampling, refer to the American Conference of Governmental Industrial Hygienists' document, "Bioaerosols: Assessment and Control."

f. Personnel conducting the sampling must be trained in proper air sampling methods for microbial contaminants. A laboratory specializing in mycology should be consulted for specific sampling and shipping instructions.

2.4 Analysis of Environmental Samples

Microscopic identification of the spores/colonies requires considerable expertise. These services are not routinely available from commercial laboratories. Documented quality control in the laboratories used for analysis of the bulk/surface and air samples is necessary. The American Industrial Hygiene Association (AIHA) offers accreditation to microbial laboratories (Environmental Microbiology Laboratory Accreditation Program (EMLAP)). Accredited laboratories must participate in quarterly proficiency testing (Environmental Microbiology Proficiency Analytical Testing Program (EMPAT)).

Evaluation of bulk/surface and air sampling data should be performed by an experienced health professional. The presence of few or trace amounts of fungal spores in bulk/surface sampling should be considered background. Amounts greater than this or the presence of fungal fragments (e.g., hyphae, and conidiophores) may suggest fungal colonization, growth, and/or accumulation at or near the sampled location. (Yang C. P&K Microbiology Services.) Air samples should be evaluated by means of comparison (i.e., indoors to outdoors) and by fungal type (e.g., genera, and species). In general, the levels and types of fungi found should be similar indoors (in non-problem buildings) as compared to the outdoor air. Differences in the levels or types of fungi found in air samples may indicate that moisture sources and resultant fungal growth may be problematic.

3. REMEDIATION

In all situations, the underlying cause of water accumulation must be rectified or fungal growth will recur. Any initial water infiltration should be stopped and cleaned immediately. An immediate response (within 24 to 48 hours) and thorough clean up, drying, and/or removal of water damaged materials will prevent or limit mold growth. If the source of water is elevated humidity, relative humidity should be maintained at levels below 60% to inhibit mold growth. (ASHRAE, 1992.) Emphasis should be on ensuring proper repairs of the building infrastructure, so that water damage and moisture buildup does not recur.

Five different levels of abatement are described below. The size of the area impacted by fungal contamination primarily determines the type of remediation. The sizing levels below are based on professional judgement and practicality; currently there is not adequate data to relate the extent of contamination to frequency or severity of health effects. **The goal of remediation is to remove or clean contaminated materials in a way that prevents the emission of fungi and dust contaminated with fungi from leaving a work area and entering an occupied or non-abatement area, while protecting the health of workers performing the abatement.** The listed remediation methods were designed to achieve this goal, however, due to the general nature of these methods it is the responsibility of the people conducting remediation to ensure the methods enacted are adequate. The listed remediation methods are not meant to exclude other similarly effective methods. Any changes to the remediation methods listed in these guidelines, however, should be carefully considered prior to implementation.

Non-porous (e.g., metals, glass, and hard plastics) and semi-porous (e.g., wood, and concrete) materials that are structurally sound and are visibly moldy can be cleaned and reused. Cleaning should be done using a detergent solution. Porous materials such as ceiling tiles and insulation, and wallboards with more than a small area of contamination should be removed and discarded. Porous materials (e.g., wallboard, and fabrics) that can be cleaned, can be reused, but should be discarded if possible. A professional restoration consultant should be contacted when restoring porous materials with more than a small area of fungal contamination. All materials to be reused should be dry and visibly free from mold. Routine inspections should be conducted to confirm the effectiveness of remediation work.

The use of gaseous ozone or chlorine dioxide for remedial purposes is **not** recommended. Both compounds are highly toxic and contamination of occupied space may pose a health

threat. Furthermore, the effectiveness of these treatments is unproven. For additional information on the use of biocides for remedial purposes, refer to the American Conference of Governmental Industrial Hygienists' document, "Bioaerosols: Assessment and Control."

3.1 LEVEL I: SMALL ISOLATED AREAS (10 sq. ft or less) - e.g., ceiling tiles, small areas on walls

a. Remediation can be conducted by regular building maintenance staff. Such persons should receive training on proper clean up methods, personal protection, and potential health hazards. This training can be performed as part of a program to comply with the requirements of the OSHA Hazard Communication Standard (29 CFR 1910.1200).

b. Respiratory protection (e.g., N95 disposable respirator), in accordance with the OSHA respiratory protection standard (29 CFR 1910.134), is recommended. Gloves and eye protection should be worn.

c. The work area should be unoccupied. Vacating people from spaces adjacent to the work area is not necessary but is recommended in the presence of infants (less than 12 months old), persons recovering from recent surgery, immune suppressed people, or people with chronic inflammatory lung diseases (e.g., asthma, hypersensitivity pneumonitis, and severe allergies).

d. Containment of the work area is not necessary. Dust suppression methods, such as misting (not soaking) surfaces prior to remediation, are recommended.

e. Contaminated materials that cannot be cleaned should be removed from the building in a sealed plastic bag. There are no special requirements for the disposal of moldy materials.

f. The work area and areas used by remedial workers for egress should be cleaned with a damp cloth and/or mop and a detergent solution.

g. All areas should be left dry and visibly free from contamination and debris.

3.2 LEVEL II: MID-SIZED ISOLATED AREAS (10 - 30 sq. ft.) - e.g., individual wallboard panels.

a. Remediation can be conducted by regular building maintenance staff. Such persons should receive training on proper clean up methods, personal protection, and potential health hazards. This training can be performed as part of a program to comply with the requirements of the OSHA Hazard Communication Standard (29 CFR 1910.1200).

b. Respiratory protection (e.g., N95 disposable respirator), in accordance with the OSHA respiratory protection standard (29 CFR 1910.134), is recommended. Gloves and eye protection should be worn.

c. The work area should be unoccupied. Vacating people from spaces adjacent to the work area is not necessary but is recommended in the presence of infants (less than 12 months old), persons having undergone recent surgery, immune suppressed people, or people with chronic inflammatory lung diseases (e.g., asthma, hypersensitivity pneumonitis, and severe allergies).

d. The work area should be covered with a plastic sheet(s) and sealed with tape before remediation, to contain dust/debris.

e. Dust suppression methods, such as misting (not soaking) surfaces prior to remediation, are recommended.

f. Contaminated materials that cannot be cleaned should be removed from the building in sealed plastic bags. There are no special requirements for the disposal of moldy materials.

g. The work area and areas used by remedial workers for egress should be HEPA vacuumed (a vacuum equipped with a High-Efficiency Particulate Air filter) and cleaned with a damp cloth and/or mop and a detergent solution.

h. All areas should be left dry and visibly free from contamination and debris.

3.3 Level III: Large Isolated Areas (30 - 100 square feet) - e.g., several wallboard panels.

A health and safety professional with experience performing microbial investigations should be consulted prior to remediation activities to provide oversight for the project.

The following procedures at a *minimum* are recommended:

a. Personnel trained in the handling of hazardous materials and equipped with respiratory protection, (e.g., N95 disposable respirator), in accordance with the OSHA respiratory protection standard (29 CFR 1910.134), is recommended. Gloves and eye protection should be worn.

b. The work area and areas directly adjacent should be covered with a plastic sheet(s) and taped before remediation, to contain dust/debris.

c. Seal ventilation ducts/grills in the work area and areas directly adjacent with plastic sheeting.

d. The work area and areas directly adjacent should be unoccupied. Further vacating of people from spaces near the work area is recommended in the presence of infants (less than 12 months old), persons having undergone recent surgery, immune suppressed people, or people with chronic inflammatory lung diseases (e.g., asthma, hypersensitivity pneumonitis, and severe allergies).

e. Dust suppression methods, such as misting (not soaking) surfaces prior to remediation, are recommended.

f. Contaminated materials that cannot be cleaned should be removed from the building in sealed plastic bags. There are no special requirements for the disposal of moldy materials.

g. The work area and surrounding areas should be HEPA vacuumed and cleaned with a damp cloth and/or mop and a detergent solution.

h. All areas should be left dry and visibly free from contamination and debris.

If abatement procedures are expected to generate a lot of dust (e.g., abrasive cleaning of contaminated surfaces, demolition of plaster walls) or the visible concentration of the fungi is heavy (blanket coverage as opposed to patchy), then it is recommended that the remediation procedures for Level IV are followed.

3.4 Level IV: Extensive Contamination (greater than 100 contiguous square feet in an area)

A health and safety professional with experience performing microbial investigations should be consulted prior to remediation activities to provide oversight for the project. The following procedures are recommended:

a. Personnel trained in the handling of hazardous materials equipped with:
   i. Full-face respirators with high efficiency particulate air (HEPA) cartridges
   ii. Disposable protective clothing covering both head and shoes
   iii. Gloves
b. Containment of the affected area:
   i. Complete isolation of work area from occupied spaces using plastic sheeting sealed with duct tape (including ventilation ducts/grills, fixtures, and any other openings)
   ii. The use of an exhaust fan with a HEPA filter to generate negative pressurization
   iii. Airlocks and decontamination room
c. Vacating people from spaces adjacent to the work area is not necessary but is recommended in the presence of infants (less than 12 months old), persons having undergone recent surgery, immune suppressed people, or people with chronic inflammatory lung diseases (e.g., asthma, hypersensitivity pneumonitis, and severe allergies).
d. Contaminated materials that cannot be cleaned should be removed from the building in sealed plastic bags. The outside of the bags should be cleaned with a damp cloth and a detergent solution or HEPA vacuumed in the decontamination chamber prior to their transport to uncontaminated areas of the building. There are no special requirements for the disposal of moldy materials.
e. The contained area and decontamination room should be HEPA vacuumed and cleaned with a damp cloth and/or mop with a detergent solution and be visibly clean prior to the removal of isolation barriers.
f. Air monitoring should be conducted prior to occupancy to determine if the area is fit to reoccupy.

3.5 Level V: Remediation of HVAC Systems

3.5.1 A Small Isolated Area of Contamination (<10 square feet) in the HVAC System

a. Remediation can be conducted by regular building maintenance staff. Such persons should receive training on proper clean up methods, personal protection, and potential health hazards. This training can be performed as part of a program to comply with the requirements of the OSHA Hazard Communication Standard (29 CFR 1910.1200).
b. Respiratory protection (e.g., N95 disposable respirator), in accordance with the OSHA respiratory protection standard (29 CFR 1910.134), is recommended. Gloves and eye protection should be worn.
c. The HVAC system should be shut down prior to any remedial activities.

d. The work area should be covered with a plastic sheet(s) and sealed with tape before remediation, to contain dust/debris.
e. Dust suppression methods, such as misting (not soaking) surfaces prior to remediation, are recommended.
f. Growth supporting materials that are contaminated, such as the paper on the insulation of interior lined ducts and filters, should be removed. Other contaminated materials that cannot be cleaned should be removed in sealed plastic bags. There are no special requirements for the disposal of moldy materials.
g. The work area and areas immediately surrounding the work area should be HEPA vacuumed and cleaned with a damp cloth and/or mop and a detergent solution.
h. All areas should be left dry and visibly free from contamination and debris.
i. A variety of biocides are recommended by HVAC manufacturers for use with HVAC components, such as, cooling coils and condensation pans. HVAC manufacturers should be consulted for the products they recommend for use in their systems.

3.5.2 Areas of Contamination (>10 square feet) in the HVAC System

A health and safety professional with experience performing microbial investigations should be consulted prior to remediation activities to provide oversight for remediation projects involving more than a small isolated area in an HVAC system. The following procedures are recommended:

a. Personnel trained in the handling of hazardous materials equipped with:
   i. Respiratory protection (e.g., N95 disposable respirator), in accordance with the OSHA respiratory protection standard (29 CFR 1910.134), is recommended.
   ii. Gloves and eye protection
   iii. Full-face respirators with HEPA cartridges and disposable protective clothing covering both head and shoes should be worn if contamination is greater than 30 square feet.
b. The HVAC system should be shut down prior to any remedial activities.
c. Containment of the affected area:
   i. Complete isolation of work area from the other areas of the HVAC system using plastic sheeting sealed with duct tape.
   ii. The use of an exhaust fan with a HEPA filter to generate negative pressurization.
   iii. Airlocks and decontamination room if contamination is greater than 30 square feet.
d. Growth supporting materials that are contaminated, such as the paper on the insulation of interior lined ducts and filters, should be removed. Other contaminated materials that cannot be cleaned should be removed in sealed plastic bags. When a decontamination chamber is present, the outside of the bags should be cleaned with a damp cloth and a detergent solution or HEPA vacuumed prior to their transport to uncontaminated areas of the building. There are no special requirements for the disposal of moldy materials.

e. The contained area and decontamination room should be HEPA vacuumed and cleaned with a damp cloth and/or mop and a detergent solution prior to the removal of isolation barriers.

f. All areas should be left dry and visibly free from contamination and debris.

g. Air monitoring should be conducted prior to re-occupancy with the HVAC system in operation to determine if the area(s) served by the system are fit to reoccupy.

h. A variety of biocides are recommended by HVAC manufacturers for use with HVAC components, such as, cooling coils and condensation pans. HVAC manufacturers should be consulted for the products they recommend for use in their systems.

4. HAZARD COMMUNICATION

When fungal growth requiring large-scale remediation is found, the building owner, management, and/or employer should notify occupants in the affected area(s) of its presence. Notification should include a description of the remedial measures to be taken and a timetable for completion. Group meetings held before and after remediation with full disclosure of plans and results can be an effective communication mechanism. Individuals with persistent health problems that appear to be related to bioaerosol exposure should see their physicians for a referral to practitioners who are trained in occupational/environmental medicine or related specialties and are knowledgeable about these types of exposures. Individuals seeking medical attention should be provided with a copy of all inspection results and interpretation to give to their medical practitioners.

CONCLUSION

In summary, the prompt remediation of contaminated material and infrastructure repair must be the primary response to fungal contamination in buildings. The simplest and most expedient remediation that properly and safely removes fungal growth from buildings should be used. In all situations, the underlying cause of water accumulation must be rectified or the fungal growth will recur. Emphasis should be placed on preventing contamination through proper building maintenance and prompt repair of water damaged areas.

Widespread contamination poses much larger problems that must be addressed on a case-by-case basis in consultation with a health and safety specialist. Effective communication with building occupants is an essential component of all remedial efforts. Individuals with persistent health problems should see their physicians for a referral to practitioners who are trained in occupational/environmental medicine or related specialties and are knowledgeable about these types of exposures.

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WEB-SITES RECOMMENDED BY AUTHORS FOR GENERAL INFORMATION AND RESOURCES

ENVIRONMENTAL NEWS AND PATIENT INFORMATIONS:

- Air Infiltration and Ventilation (AIVC) ..... www.aivc.org
- American Lung Association:..... www.lungusa.org
- American Type Culture Collection Home Page (Commercial)..... www.atcc.org/
- ASHRAE American Society of Heating, Refrigerating and Air Conditioning Engineers .. .. www.ashrae.org/
- Asthma & Allergy Foundation of America: ..... www.aafa.org
- Chemistry Laboratory (Bruce B. Jarvis) ..... www.chem.umd.edu/organic/jarvis.html
- California Air Resources Board Indoor Air Quality Projects .... arbis.arb.ca.gov/research/apr/past/indoor.htm
- California Indoor Air Quality Program ..... www.cal-iaq.org/
- Canada Mortgage and Housing Corporation (CMHC): ... www.cmhc-schl.gc.ca/cmhc.html
- Carpet and Rug Institute..... www.carpet-rug.com/
- Combating mold and mildew in homes from Family Handyman (General Public)..... ..... www.familyhandyman.com
- Danish Building Research Institute ..... www.sbi.dk/
- DANVAK Danish Society of Heating, Ventilating and Air-Conditioning Engineers .... www.danvak.dk/
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PHOTOS

Alternaria alternata, EM 4,200 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 25

Alternaria, EM 2,100 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 111

Aspergillus fumigatus, EN 3,300 x, ml 10 mm, dry wall, San Diego 1998, (E. JOHANNING, P.H. SIERCK); M. GAREIS, Institute for Microbiology and Toxicology, Federal Centre for Meat Research, D-95326 Kulmbach, Germany. 213

Aspergillus heads, EM 580 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 492

Aspergillus, EM 10,600 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 200

Chaetomium fumigatus, EM 2,300 x, ml 10 mm, gypsum board Albany, 1998, (E. JOHANNING, P.H. SIERCK); M. GAREIS, Institute for Microbiology and Toxicology, Federal Center for Meat Research, D-95326 Kulmbach, Germany . . . 572

Cladosporium conidia, EM 5,400 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 37

Deteriorated wool fiber cuticle, SEM (2000x). Cuticle particulates, about 30,000 /m³, many respirable, were observed in an air sample in the carpeted room. Jeffrey C. MAY, J. May Home Inspections, Inc., Cambridge, MA. 558

Exposed spermatozoa stained with calcein-AM and ethidium homodimer-1. Epifluorescence microscope photographs. Magnification 2000x. Joanna PELTOLA, University of Helsinki, Department of Applied Chemistry and Microbiology, Division of Microbiology, Finland 438

Color Plates on page 671

Friable wool cortex "strands," SEM, (1000x). Joanna PELTOLA, University of Helsinki, Department of Applied Chemistry and Microbiology, Division of Microbiology, Finland 559

Massive fungal growth on wall surfaces and ceramic tiles at the basement. Ling-Ling HUNG, US Public Health Service, Division of Federal Occupational Health, Philadelphia, PA. 277

Molecular model of valinomycin. 368

Color Plates on page 671

PCR of various fungal DNA's with *Stachybotrys* specific primers. Neil A. STRAUS, Department of Botany, University of Toronto, Toronto, Ontario, Canada 348

Penicillium chrysogenum, EM 5,800 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 474

Penicillium glabrum, EM 8,000 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 296

Southern blot analysis for presents of the trichodiene synthase gene. Neil A. STRAUS, Department of Botany, University of Toronto, Toronto, Ontario, Canada 347

Stachybotrys atra, EM 3,300, ml 10 µm, dry wall, Source: San Diego, 1998, (E. JOHANNING, PH. SIERCK); M. GAREIS, Institute for Microbiology and Toxicology, Federal Center for Meat Research, D-95326 Kulmbach, Germany..... 571

Stachybotrys chartarum, EM 7,000 x, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands 199

Stachybotrys chartarum, EM, Nicole NOLARD, Institute of Public Health (IPH), Brussels, Belgium..... 53

Thin section of middle piece of boar spermatozoa exposed to methanol soluble solids of toxic building material, HMR F. Joanna PELTOLA, University of Helsinki, Department of Applied Chemistry and Microbiology, Division of Microbiology, Finland..... 436

Thin sections of bacterial cells of the CMN taxons found in gypsum liners from indoor walls from a children's day care center. Maria ANDERSSON, Dept. of Applied Chemistry and Microbiology, Helsinki University, Finland..... 399

Thin sections of middle piece of boar spermatozoa exposed (2... 6 d) to fractions of methanolic extract from *Stachybotrys chartarum* strain HMRF-4. Joanna PELTOLA, University of Helsinki, Department of Applied Chemistry and Microbiology, Division of Microbiology, Finland..... 437

Thin sections of water damaged gypsum liner. Panel A shows a complex microbial community of bacteria and fungi inhabiting the gypsum liner. Panels B and C show thin sections of bacterial cells in the gypsum liner. The figures show cell wall structures and cell division typical for actinomycetes. Maria ANDERSSON, Dept. of Applied Chemistry and Microbiology, Helsinki University, Finland..... 398

Transmission electron micrographs of human NK cells. M.S SALKINOJA-SALONEN, Dept. of Appl. Chem. & Microb., Univ. of Helsinki, Finland, Saratoga, 1998 370

Wallemia sebi, EM 1,800, Robert A. SAMSON, Centraalbureau voor chimmelcultures, Baarn, The Netherlands..... 26

AUTHORS

Ahmed, Mehboob U.; 516
Ammann, Harriet M.; 84, 520
Andersson, Maria; 359, 396, 432
Anthes, M.; 444
Auger, Pierre L.; 131
Bader, Howard A.; 523
Baker, Dean B.; 386
Barry, Brenda E.; 538
Bartlett, K.H.; 240
Beaudet, Alain; 611
Beijer, Lena; 139, 190
Biagini, Raymond E.; 320
Blanchette, Gerard; 512
Bouchard, Rémi; 131
Bowers, Holly A.; 516
Brauer, M.; 240
Brennan, Terry; 512
Breum, Niels O.; 119, 544
Bünger, J.; 112, 418
Burge, Harriet A.; 306
Bux, S.; 375
Carlson, Neil; 245
Casteleyn, L.; 594
Caulfield, Steve M.; 512
Chung, Joe; 254
Chuturgoon, AA.; 375
Coenen, Gerard J.; 119
Cole, Cari J.; 516
Cole, Eugene C.; 516
Cook, Carl E.; 516
Cooley, J. Danny ; 403
Corbett, April L.; 516
Cormier, Yvon; 258
Craner, James; 146, 158
Cross, Edward H.; 595
Curtis, Luke; 254
Dales, Robert, 298
Danaviah, S.; 375
Dearborn, Dorr; 79
Dietrich, R.; 202, 482
Dill, B.; 240
Doyon, Julien; 131
Duchaine, Caroline; 258
Dulaney, Pamela D.; 516
Dutton, M.F.; 375
Ebbehøj, Niels; 119, 544
Etzel, Ruth A.; 79
Exuzides, K. Alexandros; 411
Fedoruk, Marion J.; 386
Gagnon, Louis; 611
Gareis, M.; 131, 202, 482
Gordon, Wayne A.; 94
Gqaleni, N.; 375
Gravesen, Suzanne; 99, 423, 505
Grigorjev, P.; 359
Haatainen, S.; 529
Haddad, Lisa; 94
Hallier, E.; 112, 418
Hansen, M.Ø.; 99
Harney, Sharon; 411
Haverinen, U.; 529
Heikkinen, Irmeli; 54
Heinsohn, Patricia; 330, 411
Helin, J.; 359
Hinkley, Simon F.; 232
Hintikka, Eeva-Liisa; 214, 221
Hirvonen, Maija-Riitta; 66
Hodgson, Mark; 268
Hodgson, Michael; 476
Horner, W. Elliott; 313

Hryhorczuk, Daniel; 254
Hung, Ling-Ling; 275
Husman, Tuula; 166, 174, 186, 195, 355, 574, 628
Hyvärinen, Anne; 166, 186, 355, 453
Ivens, Ulla I.; 119
Jarvis, Bruce B.; 221, 232
Jenkins, Constance L.; 287
Jensen, H.L.C.; 99
Jesenská, Zdenka; 351
Johanning, Eckardt; 12, 70, 94, 202, 482, 628
Jorgensen, O.; 544
Juhani, Kangas; 381
Jumper, Cynthia A.; 403
Karttunen, Riitta; 54
Kasaner., Jukka-Pekka; 60, 106
Kauriinvaha, E.; 529
Keller, F..; 532
Kelley, Robert; 611
Kennedy, S.M.; 240
Keski-Karhu, J.; 355
Kettunen, A.-V.; 529
Kjær, Birgitte; 119
Koivisto, J.; 355
Koljalg, U.; 359
Korpi, Anne; 60, 106,
Korppi, M.; 186
Kurup, Viswanath P.; 620
Landsbergis, Paul; 70
Lappalainen, Sanna; 465, 541, 616
Larsen, L.; 99
Lax, Michael B.; 125
Leese, Keith E.; 516
Lehrer, Samuel B.; 313
Lignell, Ulla; 66
Lindroos, Outi; 465, 541, 616
Ludwig, Jerry F.; 538
Lund, Thomas; 119
MacDonald, J.; 444
Mainville, Claude; 131, 611
Manetti, Federica A.; 125
Marinkovich, Vincent A.; 169
Märtlbauer, E.; 482
Mason, C.D.; 444
May, Jeffrey C.; 554
McCarthy, John F.; 538
McGuinness, Michael; 603
McGuinness, Patrick D.; 603
McNally, Daniel; 476
Meklin, Teija; 166, 186, 355, 453, 529
Mériaux, Anne; 258
Midtgaard, Uffe; 119, 544
Mikkola, Raimo; 359, 432
Miller, J. David; 131, 298
Moffett, Patrick; 457
Morey, Philip R.; 494
Müller, M.; 112, 418
Mussalo-Rauhamaa, Helena; 359, 432
Nevalainen, Aino; 66, 166, 186, 195, 355, 453, 529, 574, 628
Newell, Michael S.; 516
Nichum, E.; 99
Nielsen, Kristian Fog; 423
Nielsen, N.; 544
Nikulin, Marjo; 465
Nolard, Nicole; 48
Ohgke, H.; 532
Okumura, Hiroki; 221
Ortiz, Carol; 476
Oulton, M.; 444
Paananen, A.; 359
Pasanen, Anna-Liisa; 60, 106, 174
Pasanen, Pertti; 60
Patnode, Francis A.; 512

Peltola, Joanna; 359, 432
Pépin, Pierrot; 131
Persky, Victoria; 254
Piecková, Elena; 351
Pinard, Marie-France; 131, 611
Pratt, Adam J.; 523
Puccetti, Andrew; 457
Quraishi, Arif; 245
Ramakrishnan, Viswanathan; 254
Rand, Thomas G.; 444, 581
Räsänen, Jouni; 195
Raunio, Päivi; 174
Reijula, Kari; 38, 465, 541, 616, 628
Reiman, Marjut; 166, 453
Reindl, Douglas T.; 179
Reponen, Tjina; 628
Robertson, Larry D.; 282
Ross, Mary; 254
Ruotsalainen, Marjo; 66
Rylander, Ragnar; 28, 139, 190
Saarinen, Lauri; 465
Sabath, Herman; 549
Salkinoja-Salonen, Mirja S.; 359, 396, 432
Salo, Päivi; 561
Samson, Robert A;. 33
Saris, N-E.; 359
Sawyer, Daryl; 494
Scheff, Peter; 254
Schneider, E.; 482
Scott, James A.; 335, 343
Scott, Richard; 268,
Selman, Laurie; 516
Senkpiel, K.; 532
Sierck, Peter H.; 287
Sigsgaard, Torben; 99, 119
Sirpa, Laitinen; 381
Siwinski, Greg; 125
Stalder, K.; 112
Stetzenbach, Linda D.; 158
Storey, Eileen; 476
Straus, David C.; 403
Straus, Neil A.; 335, 343
Suadicani, Poul; 119
Sugamata, Masao; 221
Sugiura, Yoshitsugu; 221
Sumarah, M.W.; 444
Suutari, Merja; 66
Taskinen, T.; 186
Thorn, Jörgen; 139, 190
Tiffany, John A.; 523
Tiilikainen, Anja S.; 54
Timonen, T.; 359
Toivola, Mika; 453
Tsai, Stella M.; 330, 457
Tsitko, Irina; 396
Tuomi, Tapani; 465
Turner, William A.; 512
Ueno, Yoshio; 221
Uhlman, Steven; 386
Usleber, E.; 482
Vahteristo, Mikko; 166, 195, 355, 529
Van Damme, K.; 594
Van den Berghe, H.; 594
VanNetten, C.; 240
Vedenpää, Kirsi; 54
Viljanen, M.; 529
Virtanen, Tuomas; 174
Vuorio, Riitta; 359
Wadden, Richard; 254
White, Jim H.; 298, 568
Wilikins, Ken; 351
Wong, Bess; 335, 343

Wong, Wing C.; 403
Würtz, Helle; 119, 544
Yang, Chin S.; 330, 457
Yang, Haiou; 386
Yoshino, Naoto; 221

Other:

New York City Department of Health
Bureau of Environmental &
Occupational Disease Epidemiology; 635

INDEX

actinomycetes, 8-9, 42, 49, 112-114, 116-118, 127, 149, 177, 187, 260, 311, 370-371, 373, 384, 396-398, 400-402, 418-420, 422, 454, 508, 530, 541-543, 563, 567, 576, 579, 603, 606, 616-618, 631, 650, 656

aerobiology, 43, 258, 306-307, 430, 463, 510

aerosols, 22-23, 125-127, 130, 181, 219, 306, 310, 375, 463, 520, 522, 560, 580, 629

agricultural dust, 254

air conditioning, 89, 159, 171, 184, 241, 283, 285, 387, 512, 515, 554-556, 560, 566, 604, 607-608, 636, 652, 654

air samples, 42, 44, 89, 126-127, 133-134, 147, 150-151, 167, 214, 248, 259, 265, 276-278, 280, 287-289, 294, 298-299, 302, 340-341, 344, 382, 384, 388, 393, 411, 418, 455, 457-458, 463, 466, 503, 524-526, 531, 534, 543, 556-557, 559, 583, 587, 599, 605-606, 616-618, 642-643

airborne fungal matter, 7, 457, 463

airborne transmission, 179-181

Air-O-Cell cassette, 457-458, 463

air ways inflammation

allergy, 15-18, 20, 38-39, 42-43, 47-49, 52, 54, 56, 59, 64, 66, 69-72, 74, 76, 87, 105, 110, 117-118, 124, 155, 169-170, 172-174, 177-178, 186, 189, 197-198, 253, 266, 304-305, 313, 315, 317-319, 328, 356-357, 380, 403, 423, 429, 507, 510, 554, 557, 560, 575-576, 578, 596, 619, 624-625, 630-631, 652-653

Alternaria, 25, 34-35, 49, 51, 72, 111, 161-162, 209, 211, 245, 249-250, 255, 265, 273, 277-278, 290-291, 314, 318-319, 341, 351-354, 389, 391, 393, 409, 423-425, 429, 431-432, 435, 502, 516, 518, 551-552, 586-587, 606, 630, 655

Andersen single-stage sampler (N-6), 159, 455, 457, 458

anti-mold IgG antibodies, 54

anti-mold immunity, 54

apartment house, 355

Aspergillus, 18, 20, 30, 33-37, 40, 42, 49, 51, 54-57, 70-74, 77, 89, 99-101, 112-113, 116, 118, 131, 133-138, 146, 149-151, 154, 156, 163, 165, 168, 172, 176-177, 187, 200, 203, 210-211, 213, 215, 218-220, 238-239, 242, 245, 248-249, 251, 255, 257, 265, 268, 271-273, 275, 277-279, 287, 289-294, 299, 302-303, 314, 316, 318-319, 328, 330, 332-333, 335-336, 341, 351-352, 363, 375, 378, 380, 389, 391, 410, 418-420, 423, 425-426, 430, 432, 435, 440, 454, 466, 470, 472, 490, 492, 494, 501-503, 505-507, 510, 516, 518-519, 521, 524-525, 528, 530, 532, 535, 541-543, 549-552, 556-557, 581-582, 586-588, 603, 606, 618, 620-625, 630-631, 635, 637, 640, 650, 655

- *Aspergillus fumigatus*, 18, 30, 33-35, 42, 49, 54-57, 72-74, 113, 118, 131, 135, 176-177, 187, 213, 215, 218-220, 249, 272, 314, 316, 318-319, 418-419, 440, 454, 518, 521, 581, 587, 620, 624-625, 640, 655
- *Aspergillus versicolor*, 20, 33-35, 56, 71, 77, 100, 133-134, 136-138, 149, 156, 165, 187, 238, 248, 272, 278, 289, 328, 333, 363, 380, 418-419, 423, 430, 472, 490, 494, 501, 503, 505-507, 510, 518, 524, 528, 541-543, 606, 618, 631, 650

asthma, 15, 17-18, 30, 38-39, 41-44, 46-49, 51, 65-69, 87, 100, 117, 119-121, 123, 129, 131-132, 135, 140, 146-147, 149-150, 152-153, 160-161, 174, 177, 186-189, 191, 194, 196-198, 299-300, 304-305, 319, 376-377, 380, 403, 409, 507, 554-555, 557, 560, 576, 578, 604, 612, 619-621, 630-631, 634-636, 638-639, 641, 644-646, 652

attorney-client privilege, 595-596

bacteria, 6, 8, 12-13, 16, 18-20, 24, 28, 30-31, 41-44, 56, 69, 77, 87, 99, 119, 123-127, 129, 132-134, 137, 164, 176, 179, 182-183, 190, 212, 214, 219-220, 232, 237-241, 254-261, 266, 274, 286, 311, 333, 359, 361-363, 368, 370-373, 380-386, 388, 390, 392-394, 396-398, 401, 424, 435, 439, 442-443, 463, 472-473, 490-491, 504, 507-508, 521, 530, 532, 538-540, 563, 565, 574, 580, 592, 603, 606, 609, 611, 613, 617-619, 630, 656,

bioaerosol, 5, 7, 12, 80, 113, 124, 153, 214, 240, 243, 245, 247-248, 251, 254, 256-257, 275, 326, 377-378, 381, 386, 388, 393, 463, 479, 494, 512, 514-515, 520-522, 544-545, 548, 554, 559-560, 629, 636, 640, 648, 650

biological contamination, 87, 131, 520

biological monitoring, 6, 320-321, 323-325, 327-329

biomonitoring, 4, 112, 320

building, 4, 7-8, 12-15, 18, 20, 33-34, 36, 41-42, 44, 46, 52, 56, 60-62, 64, 66, 70-71, 73, 76-78, 87, 89, 91, 99-105, 128, 132-137, 146-147, 151-168, 177, 187, 195-198, 202-206, 208, 210, 233, 238, 240-243, 245, 251-253, 268, 270-275, 278, 281, 283, 286-287, 298-302, 304, 307-312, 314, 330-331, 333, 335-336, 341, 343, 356, 359-364, 368, 370-372, 374-376, 380, 386-388, 391, 393-394, 396-397, 401, 403-404, 409-411, 416-417, 423-424, 426, 429-436, 440-442, 453-455, 466, 471-472, 482-483, 485-488, 490, 494-507, 509-510, 512-514, 516, 523-529, 531-532, 536, 538-540, 542, 549-554, 561-567, 569, 574, 576-577, 579, 582-592, 598, 604-608, 611-615, 617-618, 628, 633, 635-649, 651-654, 656, 672

building materials, 7, 13, 41-42, 44, 60, 64, 70-71, 78, 87, 146-147, 152, 154, 159, 162-164, 166-167, 187, 202-205, 298-302, 309, 311-312, 330-331, 335, 343, 359-361, 368, 370, 372, 375-376, 380, 396-397, 401, 411, 416-417, 423-424, 426, 429-435, 441-442, 466, 472, 482-483, 485-488, 490, 494, 500, 505-507, 509-510, 516, 524, 529, 532, 561-562, 567, 585, 607-608, 618, 636, 639-641, 672

building-related illness, 4, 146, 153, 311

Ca++ ion, 221, 223, 228

carcinogen, 298, 418, 421

carpet, 5, 48, 149-150, 204, 268-269, 273-274, 276, 280, 336, 434, 497, 506, 554-555, 557-558, 583, 605, 613, 652

carpet dander, 554, 558

carpet dust, 5, 48, 268-269, 273-274, 336, 613

caspases, 221, 229-230

chaetoglobosin, 35, 423, 428

Chaetomium globosum, 34-35, 51, 363, 423, 425, 505, 508

chlorine dioxide, 275, 280, 549, 551, 643

chronic respiratory symptoms, 351, 353

ciliostatic effect, 351, 353-354

ciliated epithelium, 351

Cladosporium, 34-35, 37, 42, 49, 52, 55-56, 72, 99, 101, 134, 150, 168, 209, 242, 248-251, 255, 265, 271-273, 275, 277-279, 287, 290-294, 314, 318, 332, 341, 351-352, 363, 378, 389-393, 444, 446, 457-458, 460-463, 466, 502, 516, 518, 530, 546, 549, 551-552, 557, 563, 586, 606, 613, 630, 655

CNS-symptoms, 99, 104

cognitive impairment, 3, 94-95, 97

colonization, 147, 152, 161-162, 170-172, 250, 360, 401, 494-503, 559-561, 620, 643

common cold, 102, 179-181, 357

composting, 5, 71, 113-114, 117, 190, 194, 254-257

consultants, 12, 46, 268, 387, 509, 595-596, 611-612, 635, 637

containment, 148, 154, 162, 280, 372, 494-503, 512-514, 524, 526, 551, 644, 646-647

cytotoxic T cell, 139

dampness problem, 186

Daubert, 595, 602

decontamination, 5, 252, 275-276, 278, 280, 496, 500, 512, 526-527, 549, 553, 569, 608, 614, 646-648

deposition and retention, 403

diterpenes, 232

DNA diagnostics, 343

DNA fragmentation, 221, 223, 225-230, 374

dose-response, 87, 99, 154, 195, 198, 323, 381

drywall, 208-211, 276-277, 287-289, 294, 326, 330, 336, 340, 343, 412, 415, 514, 555, 564, 566, 581, 583, 588

duct cleaning, 275, 554

dust, 5-6, 13, 28, 34, 38-39, 41, 47-48, 51, 53, 99, 104-105, 112-113, 116-124, 132-134, 136-137, 148, 157, 163, 165, 170, 188, 190-191, 193-194, 202, 214, 254-260, 263, 266, 268-269, 273-274, 298-305, 314, 316, 319, 335-336, 340-342, 360-363, 365, 368, 370, 384-385, 387, 391, 393, 397, 400, 402, 410, 420, 429, 465-471, 479, 481, 494, 497-499, 501, 506, 510, 524-525, 528, 541-547, 551-552, 554-557, 559-560, 564, 567, 578, 581-585, 611, 613-614, 631, 635, 637, 640, 643-645, 647, 650-651, 672

electron microscope, 221, 228, 397, 454

endotoxin, 8, 29, 31, 119, 123, 125-127, 129, 139, 141, 143-145, 190-194, 254-256, 258, 260, 264, 266, 299, 304, 381-385, 424, 521-522, 544-548

environment, 6, 11-13, 19-20, 22, 29-31, 38, 40-42, 46-49, 51-53, 56, 59, 64-65, 67, 69, 78, 82-83, 92, 104, 110, 129, 135, 137, 144, 146-147, 151-152, 154-156, 159-160, 165-166, 168-169, 174, 176-177, 179-180, 182-186, 194, 203, 213-215, 230, 238, 240, 243, 266, 282-285, 290, 294-295, 304, 309, 311, 314-317, 328, 330-331, 333, 335-336, 341-342, 350-351, 353, 359, 364, 371-373, 375-377, 379-381, 383, 385, 387, 396-397, 401-402, 429-430, 442-443, 457, 465, 471-472, 477, 491, 506, 510, 513, 515-517, 519-520, 522, 525, 528, 541, 547, 552-553, 555, 567, 575, 577, 579, 592, 595, 606-608, 612, 616, 619, 630, 636, 638, 650, 652-654

environmental biological exposure, 258

environmental disease, 146, 152, 158, 282-283, 476

enzyme-linked immunosorbent assay, 167, 174-175, 191, 483, 491

epidemiology, 3, 9, 12, 27-28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90-92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194-196, 198, 240, 254, 304, 307, 570, 574-575, 633-635, 649-650

expert witness testimony, 595

exposure, 3-9, 12-13, 16-20, 28-31, 38-42, 44, 46-47, 54, 59-62, 64-66, 68, 70-94, 97, 99, 101, 104-126, 128-133, 135-144, 147, 151-156, 159, 161-179, 182, 185-186, 188-198, 201-204, 206, 208, 210, 212-220, 222, 224, 226, 228, 230, 232, 234, 236, 238-240, 242, 244-248, 250-252, 254, 256-260, 262, 264, 266, 268, 270, 272, 274-276, 278, 280, 282-286, 288, 290, 292, 294, 298-300, 302-304, 306-312, 314-328, 330-331, 333, 343, 350, 355-357, 360, 369-371, 373, 375, 381-385, 387-388, 396, 404, 408-409, 412, 418, 422, 424, 430, 433, 436, 438, 443-447, 450-451, 470, 472, 476-483, 488-491, 494, 504-507, 509-510, 512, 515, 521-522, 532-536, 538-540, 544-548, 564, 567, 569-570, 574-581, 589-591, 594, 598, 603, 606, 628-632, 635-642, 648, 650-651, 653

exposure assessment, 5, 9, 12, 70, 73, 76, 118, 120, 123-124, 126, 129, 147, 154, 163, 201-204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 298, 538-540, 628-630, 632

fever, 39, 41, 72, 99, 102, 104-105, 148, 160, 171-172, 179, 214, 440, 538, 540, 557, 621, 630, 640

final clearance, 275, 280-281, 523-528

Frye, 595, 598, 602

fungal contamination, 7-8, 20, 37, 52, 92, 144, 147, 152, 155, 202, 205, 245, 250, 252-253, 268, 273, 279, 282, 298, 303, 306, 330, 350, 442-443, 458, 494, 496, 504, 526, 549, 553, 561, 566, 592, 603-604, 611, 614, 635-638, 641, 643, 648

fungal exposure, 3-4, 6, 16, 70, 72, 74-75, 77, 166, 170, 176, 314-315, 318, 320, 333, 373, 430, 603, 630-631, 635, 638-639, 641-642

fungal genera, 5, 153, 242, 255, 277, 279, 287, 289, 291-292, 314, 432

fungal growth, 5, 23, 30, 33, 71, 76, 161, 164, 186, 202-203, 205-207, 209, 211, 245, 247, 249, 251, 253, 269, 273, 275, 277, 280, 287, 289, 294, 306, 379-380, 386, 393, 411, 415-416, 494-495, 498-499, 501, 506, 517, 524, 543, 556, 561-565, 567, 589, 608, 613, 615, 636, 638, 641, 643, 648, 655

fungal spores, 31, 81, 113, 147, 159, 162, 169-170, 214-215, 218, 232, 245, 247, 251, 255, 269, 273, 286-287, 335, 341, 409, 424, 430, 450, 457-458, 494, 501-502, 510, 519, 521, 532, 535, 541-543, 556, 561, 567, 580, 592, 616-619, 638-639, 643

fungi, 1-6, 8-10, 12-13, 16-20, 22-24, 29-31, 36-39, 41-44, 47-49, 51-53, 59, 66, 69-80, 82-83, 85, 92, 94, 112-113, 115, 123-124, 126-127, 131-132, 137-139, 143, 146-159, 162-174, 176-177, 179, 182-185, 187, 189-190, 202-203, 207-209, 212-216, 219-220, 232-233, 237-243, 247-249, 251, 253-257, 268-271, 273-278, 280-281, 284-287, 291, 294-295, 298-300, 302-306, 311, 313-319, 325-326, 328, 330, 332-333, 335-336, 341-342, 347, 350-354, 359, 363, 370-371, 373, 379-380, 386, 388-395, 397-398, 403, 409-410, 417-419, 424-426, 430-432, 435, 439, 441, 443, 456-458, 463, 466-467, 470-473, 485, 490-491, 494-496, 499, 501, 503-504, 506-508, 510, 516-519, 521, 523, 525-526, 529-531, 536, 541-542, 544-547, 549-551, 553, 556, 561, 563, 565-567, 579-580, 586, 591-592, 603, 606, 609, 615-619, 628, 630-633, 635-643, 645, 650-653, 656

fungus, 6, 30, 71, 81, 91, 116, 156, 159, 164, 172, 174, 209, 215, 219, 223, 233, 236-237, 253, 309, 318, 326, 330-331, 343-344, 347, 352, 403, 418, 421, 424, 432, 485, 534, 552, 589, 605, 618, 620-621, 652, 654

Fusarium, 33-35, 42, 49, 55-56, 71, 92, 131, 134, 136, 149, 187, 203, 209, 213-214, 217, 219-220, 223, 230, 265, 273, 290, 325, 343-345, 347-350, 418, 441, 443, 454, 466, 470, 472, 491, 516, 518, 549-552, 630-631, 635, 637

Fusarium incarnatum, 131, 134, 136
glass, 8, 107, 119-124, 235, 259, 337, 342, 345, 376, 382, 497, 544-547, 555, 643
glass particles, 119, 124, 544, 546-547
glucans, 13, 16, 31, 139, 144, 193, 254, 256, 629
(1→3)-β-D-glucan, 28, 29, 31, 32, 87, 89, 100, 105, 139-141, 143-145, 190-194, 302, 303, 424, 510
gram-negative bacteria, 125, 127, 381-384
growth conditions, 3, 30, 33-34, 66, 68, 76, 170, 315-316, 411, 418-420, 485
guidelines, 9, 31, 36, 131, 184, 192, 240, 243, 281, 285, 302, 307, 328, 380, 441, 443, 494-496, 498, 500-501, 504, 512-513, 515, 520, 522-523, 570, 574, 577-579, 616, 618-619, 633, 635, 637-638, 643, 648-649
health, 1-6, 8-22, 24, 27-28, 30-34, 36-48, 50, 52-56, 58-60, 62, 64, 66, 68-78, 80, 82-84, 86-94, 96, 98-100, 102, 104-106, 108, 110, 112-114, 116-120, 122-126, 128-132, 134, 136-138, 140, 142, 144, 146, 148, 150, 152, 154-166, 168-170, 172, 174, 176-180, 182-186, 188-190, 192, 194-196, 198, 202-204, 208, 212-213, 219-220, 230, 232-233, 235, 238-240, 244, 246, 253-254, 257-258, 266, 268, 274-276, 278, 280-281, 286, 298-300, 303-306, 310, 312, 319-321, 323-326, 328, 333, 343, 350-351, 353, 355-361, 371, 373-377, 380-381, 386, 394-396, 400, 403, 410, 416, 422, 429-430, 432-434, 441-443, 453, 456, 463, 465-467, 470-473, 476-477, 481-483, 488, 490-491, 495-496, 500, 503-510, 512, 516, 519-523, 528-529, 531, 535-536, 538, 540-541, 544, 547-549, 561, 567-570, 573-580, 582, 584, 586, 588-592, 596, 598, 603-604, 606-608, 611-612, 614-617, 619, 628-633, 635-641, 643-656
health effects, 1-3, 9-10, 12, 20, 27-28, 30-32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68-72, 74, 76-78, 80, 82, 84, 86, 88, 90-92, 94, 96, 98, 100, 102, 104-106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130-132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176-180, 182, 184, 186, 188, 190, 192, 194-196, 198, 212-213, 238-239, 254, 257, 266, 299-300, 303-304, 310, 324-326, 333, 355-358, 375, 381, 416, 430, 432, 443, 463, 495, 507, 509-510, 520-522, 528, 535-536, 547, 561, 568-569, 574-577, 579, 592, 598, 603-604, 606-607, 628-633, 635-640, 643, 650-651
health risk, 19, 38, 189, 202, 208, 235, 305, 323, 538, 544, 577
hemorrhage, 3, 20, 77-83, 92, 156, 184, 219, 230, 233, 238, 310-311, 328, 333, 350, 417, 430, 490, 510, 594, 636, 638, 640, 642, 650-651
heteroduplex mobility assay, 335, 337, 342
HL-60 cells, 221, 225-226
house dust, 6, 28, 34, 188, 302, 304-305, 319, 335, 342, 384-385, 554-557, 559-560
house dust mite, 28, 554-555, 557
household waste, 4, 119-120, 123, 190-191, 546-547
houses, 3-4, 8, 17, 31, 34, 41-44, 46, 48-49, 51, 54, 59, 66, 69, 82-83, 106, 139-140, 146, 148, 152, 155, 166-168, 198, 253, 281, 286, 299-300, 303-304, 311, 333, 335-336, 338, 340-341, 351, 353, 356, 358, 380, 395, 417, 430, 432-433, 441, 443, 472, 504, 507-508, 510, 554-555, 562-564, 566-568, 570, 574-576, 579-580, 592, 594, 611, 618
housing conditions, 105, 574
hypersensitivity pneumonitis, 28, 31, 38-39, 47, 87, 105, 112, 118, 146, 151, 156, 479, 554-555, 625, 631, 635, 637, 641, 644-646, 651
hypochlorites, 549, 551

IAQ during renovation, 512

IgA, 122-123, 174-177, 622

IgE, 28, 42-43, 46-48, 70, 72, 74, 76, 122, 124, 135, 141, 153, 156, 164-165, 169-170, 173-176, 178, 191, 313-315, 317-319, 403, 406-407, 409, 424, 507, 620-625

IgG, 29, 39, 46, 48, 54-59, 70, 72-74, 76, 104, 112-117, 149, 153, 156, 164-167, 171, 173-178, 218, 434, 439-441, 507, 547, 620, 622

IgG antibodies, 29, 39, 46, 54-55, 59, 70, 112, 114, 116, 153, 166-167, 218, 434, 439, 441, 507, 547

IL-4, 403, 406-407, 409, 620, 623-624

immune complexes, 169, 171-173

immunopathology of fungal reactions, 169

immunosuppressants, 232-233

indicator fungi, 523, 526

indirect immunofluorescence, 112-113

indoor, 3-4, 6, 8-9, 11-16, 18-20, 24, 30-31, 33-38, 41-44, 46-49, 52-53, 59-65, 69-73, 75-79, 82, 92, 104-106, 108, 110, 120, 130-131, 137-138, 144, 146-147, 151, 154-156, 158-160, 164, 168, 177, 179-180, 182-184, 187, 194-195, 202-204, 209-210, 212-215, 219-220, 230, 232, 238-245, 248, 251-253, 274-278, 280-286, 302-304, 306, 309, 314-315, 318-319, 330, 333, 335-336, 340-344, 351, 353, 355, 358-361, 363-365, 368, 370-376, 378-380, 386-387, 391, 393-397, 399, 401-403, 409-412, 416-417, 423, 429-432, 434, 441-443, 453-457, 464-473, 476, 482, 485, 490-491, 494-496, 501, 504-506, 508, 510-513, 515-517, 519-520, 522, 524-526, 528, 531-537, 541, 543, 547, 549, 553, 561, 566-567, 570, 574, 576-580, 584, 586-587, 592, 597, 605-609, 612-613, 616, 618-619, 628-631, 635-639, 641, 650, 652-654, 656, 672

indoor air, 3, 11-12, 14, 18-20, 24, 31, 38, 41-44, 46-47, 59-65, 69-70, 77, 79, 82, 104-106, 108, 130-131, 137-138, 144, 156, 158-159, 164, 168, 177, 179, 184, 187, 194, 212-214, 219-220, 230, 238-241, 243-245, 252-253, 274-275, 285-286, 302-304, 330, 333, 335-336, 340-342, 344, 358, 361, 365, 371-374, 380, 394, 401-402, 410-411, 416-417, 429-430, 432, 441-443, 453-455, 464-465, 470-472, 476, 490-491, 494-496, 501, 504, 508, 510-513, 516, 519, 524, 526, 528, 531-532, 535-537, 541, 543, 561, 566-567, 570, 574, 576-580, 592, 607, 612, 616, 618-619, 628, 630-631, 650, 652-654

indoor air concentration, 60-61

indoor air pollution, 156, 164, 373, 443, 519, 561

indoor air quality, 12, 14, 20, 41, 43-44, 60, 64-65, 108, 130, 158-159, 184, 194, 240-241, 243-245, 252-253, 275, 285-286, 303, 333, 335, 340, 394, 401-402, 411, 416-417, 429, 464, 472, 495-496, 504, 508, 510-513, 528, 531, 543, 567, 574, 577-579, 607, 619, 628, 650, 652-654

indoor building materials, 309, 360, 368, 396-397, 401, 434, 672

indoor contaminant, 154, 396, 403

indoor environment, 6, 13, 41-42, 46, 52, 59, 64, 110, 144, 146, 179-180, 182-184, 203, 213, 230, 243, 285, 304, 314-315, 330, 336, 341, 351, 353, 359, 364, 371-373, 376, 379, 397, 401-402, 442, 457, 471, 491, 506, 513, 515-516, 522, 553, 607, 612, 638, 653-654

industrial hygiene, 8, 72, 77, 131, 137, 185, 243, 253, 274, 281, 285-286, 295, 300, 304, 328-329, 333-334, 395, 431, 463, 523, 592, 596, 642, 650-651

infant, 9, 79-82, 330-331, 343, 374, 593-594

inhalation, 4, 13, 16, 18, 22, 29-31, 39, 41-42, 87, 89, 92, 100, 105-107, 112, 139-140, 144, 177, 193-194, 215-220, 232, 235, 238, 258, 307-308, 310-311, 316, 320-321, 343, 375, 379, 381, 403, 407-409, 411-412, 416, 418, 445, 452, 483, 507, 509, 536, 538-539, 635, 638

intelligence, 94, 133

intervention study, 157, 165, 574

learning, 9, 12-13, 18, 94-95, 97, 133, 611

legionella bacteria, 8, 538-540

legislation, 361, 574, 577

lung, 17, 19, 29, 39-41, 46-47, 69, 79, 83, 87, 92, 105, 117-121, 139, 143, 152, 155, 168, 170, 176-178, 214-220, 230, 235, 239, 256, 258, 310, 322-323, 329, 385, 404, 433, 443-447, 451-452, 480-481, 507, 575, 579, 620-621, 623, 635, 637, 639, 641, 644-646, 650, 652, 654

lung mycotoxicosis, 83, 214, 217-219, 239, 329, 443

lymphocyte stimulation by mold antigens, 54

mail sorting, 544-546

mechanism's of hypersensitivity

media selection, 330

membrane damage, 360, 432, 436, 438

Memnoniella, 83, 232, 236, 238, 328, 331, 334, 344, 347-348, 433, 440, 491, 518, 635, 637

memory, 56, 70, 87, 94-95, 97, 133, 135-136, 149, 152, 158, 160

mice apoptosis, 221

microbes, 6, 8, 22, 38, 41-43, 46, 66, 68-69, 126, 168, 187, 198, 300, 302, 359-360, 363, 365, 370, 432-433, 435, 453-455, 464, 504, 529-531, 541-542, 553, 567, 575, 577-578, 580, 613, 629-630

microbial abatement, 523, 525, 527-528, 549

microbial aerosol control, 512

microbial contamination litigation, 9, 593, 595, 602

microbial volatile metabolites, 3, 60, 62, 64, 106

microbial volatile organic compounds (MVOCs), 64, 87, 110, 245, 386, 521, 532, 536

microorganisms,, 112-113, 123, 125, 129, 164, 174, 177, 180, 195, 259, 300, 305, 311, 333, 355, 364, 372-373, 385, 430, 435, 441, 443, 472, 504, 507, 510, 520-521, 525, 541, 544-545, 548-549, 552-553, 566, 576, 580, 604-606, 617, 629, 650-651

microscopic fungi, 6, 351

mildew, 8, 516-519, 652

moisture, 4, 6, 8, 12-15, 18, 20, 22, 34, 38-39, 41-44, 46, 60-61, 64, 72-73, 78, 84, 87, 91, 162, 166, 168, 186-189, 195-198, 207, 242, 253, 269, 287, 289, 298-299, 302-303, 311, 335, 340-341, 355-358, 360-361, 368, 391, 393-395, 432, 443, 453-454, 466, 476-478, 494-496, 498-501, 503-504, 506, 508, 510, 512, 516-519, 529-531, 538-539, 542, 552-555, 559, 561-562, 564-569, 574-580, 587, 589, 603, 605, 607-608, 616-619, 628, 630, 633-634, 636, 641, 643, 672

moisture control, 393, 561, 566-567

moisture intrusions, 603

moisture problem, 38-39, 41-44, 46, 60, 166, 186-187, 360, 368, 478, 501, 503, 608, 633-634, 672

moisture problems in school buildings, 186

mold allergens, 313, 505

mold exposure, 3, 28, 31, 38, 41, 88-89, 112, 114, 137, 143, 154, 164, 186, 188, 300, 304, 355, 418, 479, 569-570, 574-578, 591, 598, 630-632

mold growth, 15, 30, 46, 132, 161, 186, 195, 299-300, 355, 389, 392-393, 499, 504-506, 508, 524, 542, 556-557, 568, 574, 577, 579, 585, 594, 604-606, 614-616, 635, 637, 641-643

mold infestation, 505-506, 632

mold problem buildings, 61, 63-64, 166, 531, 567, 579, 619

mold-induced cytokine excretion in vitro, 54

MVOC, 8, 30, 60, 62, 106-110, 147, 245, 247-248, 252, 386, 388, 390-391, 393-394, 508, 532-536, 629

mycobacteria, 6, 370-372, 374, 396-397, 400-402

mycotoxicosis, 5, 77, 83-84, 86, 89, 137, 174-175, 214, 216-220, 239, 282, 285, 326, 328-329, 424, 429, 443, 510, 650

mycotoxin, 5-6, 30-31, 70, 73-75, 77-78, 84, 87-89, 202-204, 207, 209-210, 214, 216-219, 232, 237-238, 282, 327, 334, 347, 353, 418-420, 422-425, 429, 471-472, 482-483, 485, 489-490, 507, 509, 521, 629, 631-632

neurotoxic, 30, 94, 98, 131, 136-137

neurotoxic molds, 94

NIOSH questionnaire, 603

nocardioform actinomycetes, 396-397

non-viable fungal growth, 245

occupational, 3, 6-14, 17, 19-20, 24, 31, 38-39, 41, 43-47, 61, 65, 70-72, 77-78, 94, 99, 104, 111-112, 114, 117-120, 124-125, 129, 135, 137-138, 146-147, 152, 154-156, 158, 160, 166, 169-170, 185, 194, 202, 212-213, 219-220, 238-240, 244, 254, 266, 268, 274-276, 281, 298, 311, 320, 333, 351, 373-374, 380-383, 385-386, 388, 418, 422, 430, 442-443, 453, 463, 465, 467, 476-482, 490-491, 504, 510, 521-522, 529, 541, 544, 546-548, 573-580, 582, 584, 586, 588-590, 592, 594, 596, 603-604, 607, 616, 628, 631-633, 635-638, 640-641, 648-650, 652-655

occupational disease, 9, 476, 635, 649, 652

organic biocide, 549, 551

organic dust, 13, 38, 41, 47, 99, 104-105, 112-113, 116-120, 124, 137, 190-191, 193-194, 420, 544, 578, 631, 635, 637, 650-651

paper, 2, 8, 13, 23, 28, 33, 48, 52, 60, 62, 71, 79, 99, 105, 107, 119-124, 130, 133, 179-180, 184, 191, 194, 202, 204-205, 207-210, 217, 235, 238, 241, 285, 298, 326, 330, 348, 360, 380, 412, 415, 434-435, 439-441, 483, 486, 495, 497-498, 501-503, 523-524, 544-548, 552, 555, 574, 604, 606-607, 641, 647

Penicillium, 6, 33-37, 42, 49, 51, 55-56, 70-74, 89, 99, 101, 112-113, 131, 133-134, 136, 146, 148-151, 154, 156, 163-164, 168, 203, 209-211, 239, 242, 248, 250-251, 255, 258, 260, 265-267, 271-273, 275, 277-279, 287, 289-294, 296, 299, 301-303, 314, 330, 332, 335-336, 340-342, 351-353, 363, 375, 378, 389, 391, 395, 403, 409-410, 418-419, 423, 425-426, 430, 432, 435, 466, 470, 474, 501-503, 505-506, 510, 516, 518-519, 530, 535, 545-546, 549-552, 556-557, 563, 581-582, 586-588, 590, 603, 606, 614, 618, 630-631, 635, 637, 640, 653, 655

Penicillium aurantiogriseum, 34-35, 131, 134, 136, 301, 395, 587-588, 590

Penicillium brevicompactum, 6, 34-35, 55, 113, 131, 134, 136, 335-336, 418-419, 425, 581

Penicillium chrysogenum, 6, 33-35, 51, 403, 423, 425, 474, 505-506, 587, 655

Penicillium crustosum, 34-35, 131, 134, 136, 418-419

polysaccharide, 28

population, 14-15, 39, 52, 70, 72, 89, 123, 137, 170, 174, 181, 196-197, 277, 300, 309, 335, 338, 355, 379, 477, 480, 525, 552, 574, 576-577, 612, 631, 640

preventing fungal contamination, 8, 561

quartenary ammonium compounds, 549

recycling plant, 257, 544-545

remediation, 8-9, 12, 20, 31, 36, 76-77, 148, 154-155, 159, 171, 252, 280-281, 307, 373, 488, 494-504, 508-509, 523, 528, 551, 554, 560, 570, 575-576, 580, 596, 611-612, 614-615, 628, 635-649, 651

research program, 124, 509, 548, 574-576, 652

respiratory, 4-5, 16-20, 22, 38-39, 42-44, 46, 48-49, 61, 64, 66, 69-73, 77, 87, 89, 91-92, 99-101, 103, 105-110, 118, 120-121, 124-126, 128-130, 137, 146, 152-154, 156, 158, 160-161, 174, 176, 178-181, 184-190, 193-195, 197-198, 212, 214-217, 219, 258-259, 285, 298-300, 303-305, 318, 324, 351-358, 373, 375-377, 387, 402, 409-410, 423-424, 430, 445, 466, 496-497, 507, 510, 516, 540, 546-547, 550, 554-555, 568-570, 574-576, 579-580, 590, 607, 611, 616, 619, 621, 630, 636, 638-640, 644-647, 649-650

respiratory health, 4-5, 20, 92, 125, 195, 298, 303-304, 355, 357-358, 376-377, 570, 616, 650

respiratory manifestation in children, 186

respiratory symptoms, 4, 46, 66, 69, 99-100, 103, 105, 120, 125, 128-129, 156, 158, 186, 188, 193, 197-198, 214, 299-300, 304-305, 318, 351, 353, 355-356, 358, 375, 377, 410, 546-547, 570, 574-576, 579, 607, 619, 639-640

sarcoidosis, 7, 476-477, 479-480

satratoxins, 35, 207, 216-217, 219, 221, 223, 226, 235, 325-326, 432-433, 439, 465, 467, 482-483, 485, 489

sawmills, 5, 166, 258-260, 265-266, 574

school, 1, 4-5, 8, 10-11, 14, 17, 19-20, 24, 53, 65, 77, 94, 99-104, 124, 137, 146, 149, 158, 169, 179-180, 183, 186-188, 208-209, 219-220, 238-241, 245-247, 252, 254, 274, 283-284, 286, 306, 333, 371-374, 380, 434, 442-443, 453-455, 463, 476, 490-491, 504, 506, 508, 529, 531, 547, 557, 561, 575-576, 578-579, 581-592, 616-619, 649

scientific evidence, 9, 595, 598, 600

sensitization to molds, 4, 186

sensory irritation, 4, 60-62, 64-65, 106-108, 110, 130, 179

serum antibody level, 174

sewage, 4, 73, 125-126, 128, 130, 564, 566

shacks, 6, 375-376, 378

sick building syndrome, 99, 146-147, 151, 153, 156-158, 164-165, 311, 343, 403, 409-410, 433, 440, 505, 574, 579, 607

sorting plant, 544

sperm cell, 364, 432, 436, 438, 441-442

spore traps, 523, 527

spores, 5-9, 16, 22-23, 30-31, 37, 42-44, 59, 66-69, 81, 83, 86, 92, 99, 113-114, 118, 131, 137, 147, 149-151, 159, 161-164, 169-170, 175, 186, 214-220, 232, 235, 239, 245, 247, 251, 253, 255-256, 269, 273, 286-287, 300-302, 309-310, 312, 314-316, 318-319, 331, 335, 340-343, 350, 353, 388-389, 391-393, 403-409, 416, 424, 426, 430, 444-446, 449-451, 454, 457-458, 463, 471, 485, 494, 496-503, 505-510, 519, 521, 527, 532, 535-537, 541-543, 546-547, 550, 552, 556-557, 561, 567, 579-580, 585, 588, 592, 616-619, 621, 629, 631, 638-639, 642-643, 650

sporicide, 549

Stachybotrys, 4-7, 9, 17-18, 20, 22, 24, 33-35, 37, 42-43, 47, 49, 52-53, 55-56, 58-59, 70-74, 77-83, 85-86, 91-92, 94, 97, 105, 137-138, 146, 149-151, 154, 156-159, 161-165, 168, 174-177, 187, 199, 202-203, 205-220, 223, 230, 232-233, 238-239, 269, 272-273, 275, 277-282, 284-285, 301, 306, 311-312, 320, 325-334, 336, 343-344, 346-352, 354, 359, 361, 363-364, 371, 373, 403, 411-412, 415-417, 424, 430-433, 435-446, 450-451, 457, 463, 465-467, 470, 472, 482-483, 485-486, 488-491, 494-495, 501-507, 510, 516, 518, 524-525, 527-528, 535, 549-553, 570-571, 575-576, 581, 587, 594, 605, 611-613, 615, 632, 635, 637, 650, 654-656

Stachybotrys atra, 20, 24, 42, 59, 70, 72-73, 77-78, 82-83, 91-92, 94, 97, 105, 177, 207, 214, 216-217, 219-220, 223, 232-233, 238-239, 281, 311-312, 328-329, 333, 354, 417, 430-431, 443, 472, 486, 490, 494, 504, 510, 528, 570-571, 594, 635, 637, 650, 655

Stachybotrys chartarum, 4-7, 9, 17-18, 20, 22, 34-35, 43, 47, 52-53, 55-56, 58-59, 71, 77-80, 83, 92, 137-138, 146, 149-151, 154, 156-159, 161-165, 174-175, 177, 199, 202-203, 205-209, 211-213, 238-239, 269, 272-273, 275, 277-280, 282, 284-285, 301, 306, 320, 326-328, 330-334, 336, 343-344, 346-352, 359, 361, 363-364, 371, 373, 411-412, 415-416, 424, 430, 432-433, 435-446, 450-451, 457, 463, 482, 488, 490-491, 494, 501, 505-507, 510, 524-525, 527-528, 551, 575, 581, 587, 605, 611-613, 615, 635, 637, 650, 656

Stachybotrys chartarum (atra), 20, 158, 159, 373, 442-443, 445, 451

steam cleaning, 554

sterigmatocystin, 35, 353, 418, 420-421, 423-424, 426, 468, 507

T-2 toxin, 83, 85, 217-218, 220-221, 223, 225, 228, 230-231, 465, 467-471, 484, 488, 490

teachers, 14-15, 73, 99-100, 104, 582

TNF-a, 55, 57, 59, 68, 139, 141-144, 191, 193, 403, 405, 407-408

toxic encephalopathy, 131

toxicity, 3, 16, 34, 66, 68-69, 78, 81, 85, 88, 134, 144, 153, 202, 206-207, 209-210, 213, 216-219, 237-238, 308, 312, 326, 334, 360, 364, 368, 371, 401-402, 418, 432-435, 439-441, 450, 482, 507, 509, 629

toxigenic fungi, 3-4, 20, 47, 52, 59, 70, 78, 83, 92, 94, 131-132, 137, 146, 153-154, 156-159, 163, 165, 177, 185, 213, 232-233, 238, 285, 328, 333, 350, 430, 443, 472, 491, 551, 586, 603, 650

tracheal organ cultures, 351-352

tremorgen, 418, 421

Trichoderma viride, 34-35, 42, 52, 55-56, 72, 207, 212, 282, 284-285, 344, 347-348, 350-352, 424, 430, 510, 541-543, 611, 613

trichodiene synthase gene, 6, 343-344, 346-347, 655

trichothecene, 7, 23, 77, 81, 83, 85, 89-91, 137, 147, 154, 156, 163, 210-211, 226, 229-231, 235, 237-239, 311-312, 329, 333, 348, 417, 429-430, 433, 439, 443-445, 450, 465-467, 470-473, 484, 490-491, 510, 599, 650

tumor necrosis factor a, 139, 403

Ulocladium, 34, 49, 52, 290, 341, 351-353, 363, 423, 425, 429, 454, 516, 518, 541-543, 563, 586-587, 611, 613

ventilation, 6, 8, 14, 33, 51, 71, 100, 140, 146, 156-159, 163-165, 170, 179, 182, 184, 240-241, 243, 259, 277, 280, 283, 285, 375, 379-380, 386-387, 394, 434, 538-539, 541-543, 545-546, 554, 561-562, 565, 567, 574, 577, 579, 581, 583, 585, 589, 606, 611, 613, 615, 617-618, 629, 636, 638, 641-642, 645-646, 652, 654

ventilation channels, 8, 541-542

verrucarins, 221, 223, 230

viruses, 4, 13, 43-44, 179-185, 232, 298

waste collectors, 4, 190-194

waste treatment, 112-114, 117, 125, 418-420

water damaged buildings, 99, 104, 204, 360, 379, 411, 416, 424, 429, 508

water damaged walls, 287

water intrusion, 13, 15, 287, 289, 294

wood dust, 258, 266, 479, 481

worker protection, 568-569

yard waste, 5, 254, 257

Color Plates

EXPOSED SPERMATOZOA STAINED WITH CALCEIN-AM AND ETHIDIUM HOMODIMER-1

FIGURE III: Exposed spermatozoa stained with calcein-AM and ethidium homodimer-1. A) Spermatozoa exposed to reagent (methanol) only and B) spermatozoa exposed (3 d) to 25 μg ml^{-1} methanol soluble substances from *Stachybotrys chartarum* HMRF-4. Spermatozoa staining green are undamaged and those staining orange have suffered damage in the cell membrane. Epifluorescence microscope photographs. Magnification 2000x.

See page 438.

MOLECULAR MODEL OF VALINOMYCIN

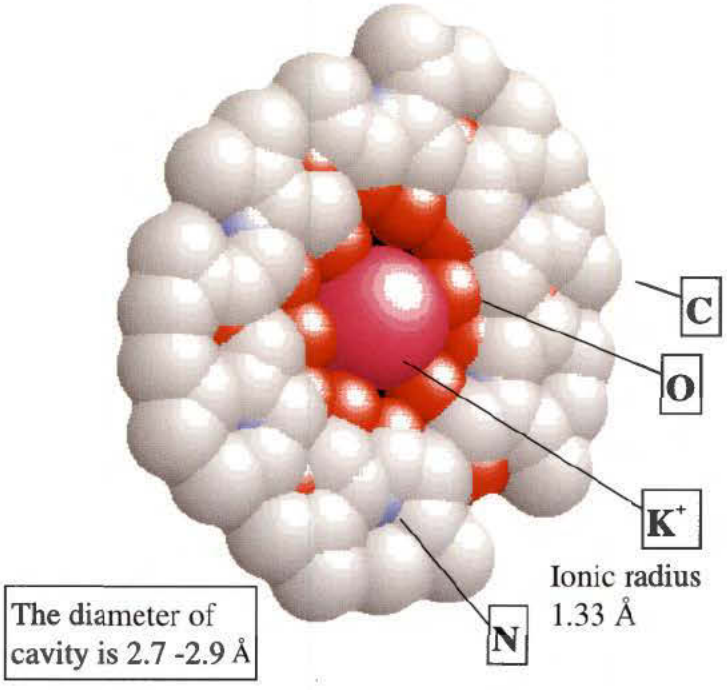
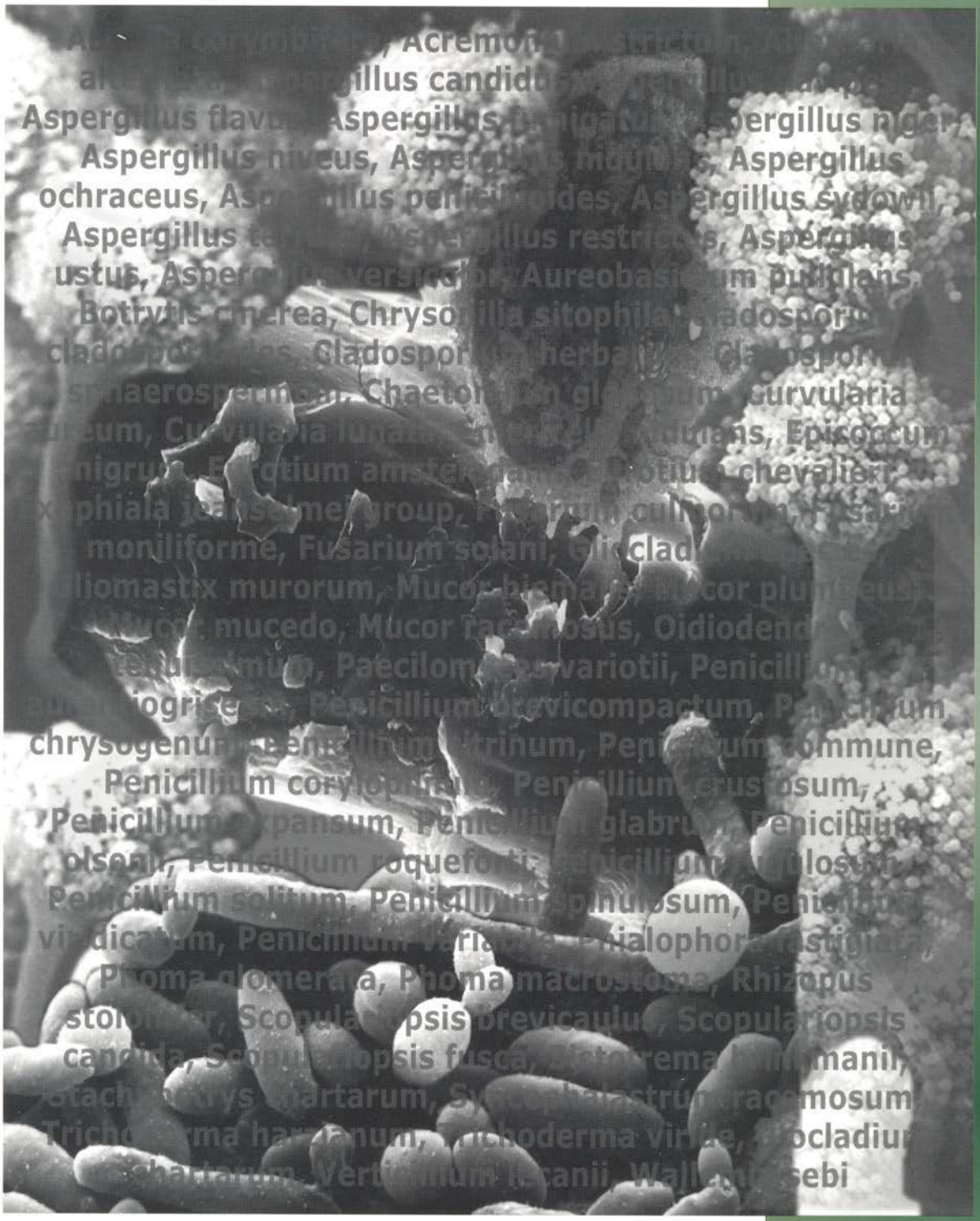


FIGURE IV: Molecular model of valinomycin, the toxin produced by several indoor strains *Streptomyces griseus*, isolated from dust and from indoor building materials of moisture problem buildings. The model shows the perfect match of the ion radius of K^+ into the channel formed in the ring-formed depsipeptide molecule, (-Val-OAla-Val-Oval-)3. The atomic diameter data were taken from Yu. Ovchinnikov (1974). Membrane active complexones. Chemistry and functions. FEBS Letters 44:1-21.

See Page 368



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