



**Bioaerosols, Fungi, Bacteria, Mycotoxins and Human Health:
Patho-physiology, Clinical Effects, Exposure Assessment,
Prevention and Control in Indoor Environments and Work**

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Bioaerosols, Fungi, Bacteria, Mycotoxins and Human Health



Dr. med. Eckardt Johanning M.D. M.Sc. (Editor)

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Fungal Research Group Foundation, Albany, New York, U.S.A.

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

International Standard Book Number: ISBN 0-9709915-1-7

Library of Congress Control Number: LCCN 2005920146

Supported by: Fungal Research Group Foundation, Albany, New York, U.S.A.

Composed by: K. J., Editing Service International, Essen, Germany

Printed by: Boyed Printing Company, Inc., Albany, New York, U.S.A.

Copyright number: PENDING

Printed in the U.S.A. on acid-free paper (1/2005)

CONTENTS

Introduction

Forword by Eckardt Johannning.....	10
Introduction by Eckardt Johannning	15
Conference Organization	17
Scientific Committee	17
Scientific coordination.....	18
Endorsement & Support.....	19

Manuscripts

Chapter 1

Health Effects I – Epidemiological Research

What do we know about dampness in buildings and health? Conclusions from two multidisciplinary reviews of the entire literature on dampness in buildings and associated health effect.....	19
Torben Sigsgaard and Carl-Gustaf Bornehag	
Mold remediation of a school and teachers' health.....	32
Riitta-Liisa Patovirta, Tuula Husman, Ulla Haverinen, Mika Toivola, Marjut Reiman, Mikko Vahteristo, Jukka Uitti, Hannu Tukiainen, and Aino Nevalainen	
Assessment of exposure to indoor environmental factors for an infant cohort at risk for asthma: preliminary analysis of bioaerosol data.....	40
Catharine M. Catranis, Susan E. Anagnost, Chun J. K. Wang, Lianjun Zhang, Analie Fernando, Shannon Morey, Paula DeStefano, Christopher Garback, Melanie LaMoy, GERALYN Hall, Judith A. Crawford, Deepa Naishadham, Andrew Hunt, and Jerrold L. Abraham	

Chapter 2

Health Effects II – Toxicology and Neurological Effects

Mold toxicity: risk assessment for humans exposed indoors	52
Harriet M. Ammann	

Cytotoxicity of different actinomycetes isolated from building materials	60
A. Mehrer, W. Lorenz, M. Gareis, C. Trautmann, R. M. Kroppenstedt, E. Stackebrandt	
Sick Building Syndrome in water damaged buildings: generalization of the chronic biotoxin-associated illness paradigm to indoor toxigenic fungi	66
Ritchie C. Shoemaker, Judith M. Rash, Elliott W. Simon	
Clinical evaluation of suspected mold neurotoxicity	78
Raymond Singer	
The chronicity of cognitive impairment associated with exposure to toxic mold	85
Wayne A. Gordon, Joshua Cantor, Heather Charatz, Teresa Ashman, Eckardt Johanning.	

Chapter 3

Health Effects III – Health Assessment & Case Studies

Health effects of moisture damage associated microbes.	94
Tuula Husman	
Laboratory signs of sickness due to mold metabolites in indoor environment	108
Anja Tiilikainen, Irmeli Heikkinen, Leila Mikkilä, and Aini Bloigu	
Assessing the allergic potential of indoor air fungal contaminants	116
Marsha D. W. Ward, Michael E. Viana, Yongjoo Chung, Najwa Haykal-Coates, Lisa B. Copeland, Steven H. Gavett, and Mary Jane K. Selgrade	
Alleged mercury poisoning symptoms in sewage treatment workers likely linked to bacteria, fungal, and endotoxin exposure	124
Chris van Netten and Karen Bartlett	
Epidemiological investigation of a mold-contaminated “sick” building	132
James Craner, Stuart Alderman and Neil Willits	
Symptomatic improvement after cessation of mold exposure: clinical experience in environmental and occupational health.	140
Iris G. Udasin, Howard Lu, Robert Laumbach, and Howard Kipen	

Chapter 4

Health Effects IV – Experimental Research

- Cytotoxicity and inflammatory responses induced by moldy house microbes 152
Majja-Riitta Hirvonen
- Do MVOCs cause irritation?: Nasal effects of VOCs and VOC oxidation products in controlled human exposures. 154
R. Laumbach, N. Fiedler, C. Weschler, C. Gardner, D. Laskin, Z. Fan, J. Zhang, P. Liroy, P. Ohman-Strickland, K. Kelly-McNeil, Y. Ma, H. Kipen
- Ocular effects in humans by experimental exposures to different types of dust. 162
Z. Pan, L. Mølhave, S. K. Kjærgaard, T. Sigsgaard, J.-E. Juto, K. Andersson and G. Stridh
- Comparison of the acute effects of *S. chartarum* and other indoor molds on the lungs of infant rats 170
I. Yike, R. Walenga and D. G. Dearborn

Chapter 5

Assessment I – Assessment Methods and Field Findings

- Exposure in moldy buildings. 180
A. Nevalainen
- Development of a gel-trap system coupled with PCR for microorganisms detection: application to Mycobacterium tuberculosis complex. 183
C. Vadrot, V. Bex, L. Bordenave, A. Mouilleseaux, V. Joseph and F. Squinazi
- House dust: an efficient and affordable tool to assess microbial contamination in homes. 191
M. F. Pinard, P. Widden, T. Debellis, C. Deblois, and C. Mainville
- Microbial Indoor Air Quality in office buildings with central air conditioning installations in Belgium. An easy tool for a fungal evaluation. 199
Camille Chasseur, Sébastien Gofflot, Nicole Nolard

Microbial Indoor Air Quality in office buildings with central air conditioning installations in Belgium. An easy tool for a bacterial evaluation.....	210
Camille Chasseur, Sébastien Gofflot, V. de Maertelaer, Nicole Nolard	
Comparison of PVC cassettes versus vacuumbags for collection and enumeration of culturable fungi in settled dust.	218
Brad Prezant	
Assessment of mold sources in indoor environments.	226
S. K. Sivasubramani, T. Reponen and S. A. Grinshpun	

Chapter 6

Assessment II – Field Findings (Continued) and Mycology

Sampling: a neglected part of building investigations and microbial exposure assessment.....	236
Anna-Liisa Pasanen	
Airborne bacteria and fungi in 100 large U.S. office buildings	240
Derek G. Shendell, Feng C. Tsai, Janet M. Macher, and Lauren Burton	
Comparison of culturable airborne fungi in water damaged buildings, bas buildings, and the outdoor air.....	246
M. S. Andrew, P. R. Morey, and B. K. Ligman	
Shared air: examining the contribution of mold from home crawl spaces to home interiors.....	256
W. Thomann, M. Miranda, A. Overstreet and M. Stiegel	
Modeling the equilibrium spore load for a building.	262
J. Banta, B. Passmore, J. Holland	
Release of <i>Aspergillus versicolor</i> fragments and spores from contaminated surfaces.....	270
Seung-Hyun Cho, Sergey Grinshpun, Mikhail Yermakov and Tiina Reponen	
Micro-particles from fungi.	276
A. M. Madsen, K. Wilkins and O. M. Poulsen	

Indoor bacilli and streptomycetes produce substances toxic to mammalian cells.	292
M. A. Andersson, E. L. Jääskeläinen, R. M. Mikkola, V. V. Teplova, P. Veijalainen, C. Apetroaie, D. Hoornstra, R. M. Kroppenstedt, M. S. Salkinoja-Salonen	

Chapter 7

Assessment III – Mycology

Fungal identification of indoor molds: current methods and new developments.....	302
Robert A. Samson	
Gypsum in nutrient medium enhances growth of <i>Stachybotrys chartarum</i>	308
A.-M. Pessi, A. Rantio-Lehtimäki	
Nutrient and pH demands of Streptomycetes isolated from moldy indoor environments.....	315
U. Lignell, M. Kontro, M.-R. Hirvonen and A. Nevalainen	
The interactions between <i>Stachybotrys chartarum</i> and <i>Streptomyces californicus</i>	322
P. Penttinen, M. M. Keinänen, M. Toivola, K. Huttunen, J. Pelkonen, A. Nevalainen, M.-R. Hirvonen	
Method to classify environmental samples based on mold analyses by QPCR.....	327
R. A. Haugland, T. Meklin, M. Varma, L. Wymer, D. Dearborn, I. Yike and S. Vesper	
Qualitative identification of <i>Meruliporia incrassata</i> using real time Polymerase Chain Reaction (PCR).....	335
K-T Lin, L. Li, D. A. Denis, R. C. Woodcock and C. S. Yang	
A new ecological niche for the pathogenic yeast, <i>Cryptococcus neoformans</i> var. <i>gattii</i> , in a temperate climate zone.....	343
K. H. Bartlett, M. Fyfe, L. MacDougall, S. Mak, C. Stephen and S. Kidd	
Individual exposure assessment in residents near large scale composting sites.	351
C. Herr, A. zur Nieden, N. Ott, W. Herzberg, J. Bünger, N. Stilianakis and Th. F. Eikmann	

Chapter 8

Assessment IV – Mycology (continued)

- Mycology. 362
L. D. Stetzenbach ·
- Cross-reactivity of monoclonal antibodies against *Aspergillus versicolor* and other fungi and their implication for the development of antibody-based monitoring techniques for fungi. 366
D. Schmechel, J. P. Simpson and D. M. Lewis
- Characterization of fungal flora from moisture damaged building material by rDNA sequencing and culture. 375
M. Pitkäranta, H. Rintala, Marja Hänninen and L. Paulin
- Fungal enzymes in indoor dust. 384
A. M. Madsen and H. Würtz
- Rapid detection and quantitation of fungal spores from dust samples using real-time PCR. 394
J. Keswani, M. Kashon and B. Chen.

Chapter 9

Transitional Countries

- The indoor environment and allergy as potential stressors in Durban hospitals, South Africa. 406
A. Shadwell, N. Gqaleni and L. Schlebusch
- Ventilation efficiency, dampness and moldiness in Durban dwellings (South Africa). 414
J. Gansan, N. Gqaleni and J. E. Ehiri
- Microbial exposure in schools studied by chemical marker analysis. Comparison between three different countries. 422
L. Wady, A. Shehabi, B. Szponar, C. Pehrson, Y. Sheng and L. Larsson
- Fungi surveys in tropical Southeastern Brazil. 429
L. S. R. Brickus, M. F. Costa, J. M. Machado and J. C. Moreira

Chapter 10

Remediation

- The effect of construction site dust on mold clearance sampling. 440
D. J. Brinkerhoff and P. R. Morey
- Assessment and remediation of mold contamination in fan coil units
of a 341-suite condominium - a case study. 447
K. White

Chapter 11

Prevention and Control

- Biological assessments focusing on mold. 456
M. Sedlar, R. Douglas and D. Pearce
- Impact of ventilation design on fungal proliferation in a school
HVAC system. 462
B. E. Barry, D. S. Jessup, T. M. Lockhart
- Mold identification and control strategies in a Canadian training
complex. 467
L. C. Nguyen Thi, C. Kerr, J. Johnson and J. Gleeson
- Risk assessment of fungal bioaerosols in indoor environments:
current paradigms and evolving concepts. 474
R. V. Miller and H. Ammann
- Sustained fungal control through interior finish performance
requirements. 482
O. C. G. Adan, M. M. Sanders and R. A. Samson

Appendix

Fotos 494

Index

FORWORD

Dear reader:

These are all the original and peer-reviewed presentations of the **Fifth International Scientific Conference on Bioaerosols in Indoor and Work Environments** held in Saratoga Springs, New York, September 10 – 12, 2003, with authors up-dates and corrections as of end of 2004. Since our first International conference and meeting in the early 90s there has been further evidence to link indoor and occupational microbial exposure which includes fungi, bacteria and their by-products with diseases of important public health implications, such as environmental allergy, asthma, inflammatory lung diseases, infections, and myco-toxins related adverse health reactions. Over time we have learned that there are many recognized biological agents and various reported adverse human health reactions, which pose great challenges to the investigators, clinicians and public health officials. Climate changes, air tight building envelopes with water infiltration and condensation problems, porous materials, inadequate building and ventilation design and maintenance, transfer of building materials and designs to countries with different climates and economical resources, and other factors appear to contribute to microbial exposure and the international concerns about its impact on the building occupants health. The intention of the scientific conference planning committee was to gather researchers and practitioners and to address the state of the art in the science and application of what recent research and practical experience has shown, to improve the assessment of microbial environmental or occupational exposure, determine important biological agents and improve the understanding and pathology of adverse human health effects, and the medical treatment, control and prevention of fungal and bacterial exposure.

One of our intended foci was to connect the experience of researchers, practitioners and consultants dealing with the “real-world” issues and concerns from different countries. Another was to encourage the participation of young investigators and from newly-developed or developing countries. The conference was a great success with more than 250 participants and representatives from more than a twelve countries. After a careful review of all submitted abstract 58 research papers were selected for the conference presentation and general discussion. Since the meeting the final papers and presentation were again critically peer-reviewed based on strict scientific criteria , revised and up-dated and are now included in this book for a general public access. The reader may please appreciate that this is not another review paper of this topic by authors with a particular motives and little scientific prior background such as can recently be found in so many periodicals, but are

truly original reports of research data and results regarding environmental exposure assessment, health and medical information, epidemiology, prevention and control techniques by authors and research groups with a demonstrated expertise in the field. The conference and meeting was supported with limited public funds. Professional educational credits were provided by the Albany Medical Center and the American Industrial Hygiene Association. The institutional sponsors were the American Industrial Hygienist Association, Fairfax, VA, the Canada Mortgage and Housing Corporation/Société Canadienne d'Hypothèques et de Logement, the University of Natal (South Africa) - Centre for Occupational and Environmental Health, the Gesellschaft für Mykotoxin Forschung, (Mycotoxin Research Association, Germany) and the International Commission Occupational Health – Organic Dust Workgroup.

Indoor air quality and occupant's health is a modern public health concern. Moisture and mold related indoor exposure and health problems in homes, offices, and public buildings (kindergartens, schools, library, and hospitals) have been gaining recognitions as one of the most common indoor environmental health issues. Although governmental and some professional organizations have recognized this, there is still inadequate general knowledge and attention among physicians regarding this health priority. The proper recognition of microbial related health problems and the differential approach are important for early and effective exposure intervention, medical diagnosis, proper treatment, referral and prevention.

In general, it is estimated that 30% or more of the buildings in the U.S.A. and Western Europe have moisture problems that can result in significant microbial contamination of the indoor environment. The combination of low-cost building materials (Paper-coated gypsum board and wood products), construction during all kinds of - weather, insufficient design consideration for moisture ventilation in energy efficient, airtight building architecture, as well as neglect of moisture intrusion, appear to be factors in the high frequency of atypical microbial exposure in buildings. The result is a multitude of health complaints and medical conditions. Some of the health complaints may also be related to other important indoor pollutants (chemical fumes, fibers, tobacco smoke, dust and mites, among others). Many of the various important biological agents that can contaminate the indoor environment such as mites, allergens, mycotoxins, endotoxins, volatile organic compounds (VOC) and microbial VOC are being investigated to improve our understanding of host factors (i.e., susceptibility) cross-reactivity, as well as the synergistic, and cumulative effects of these agents. People who get sick in such environments may be told by their physicians that they to suffer from either so-called "sick building syndrome" or "building related disease." The term *sick building syndrome* (SBS) refers often to so-called non-specific symptoms (primarily sensory complaints) that are temporally related to a problem building, but in most cases no

specific causative agent can be identified and laboratory testing often is inconclusive or negative. SBS is typically a multi-factorial problem that includes building factors, ventilation system problems, odor complaints, work-organization, and job stress and dissatisfaction, among others. The term *building-related disease or illness* (BRI) relates to diagnoses that are related to an identifiable exposure agent in a building and illnesses have distinct clinical findings with laboratory or imaging test abnormalities. The building-related illnesses can occur because of infectious, allergic-immunological, or irritant- or toxic exposures. The medical conditions and illnesses associated with microbial indoor exposure include a spectrum of infectious and respiratory disorders (bronchitis, asthma and allergic alveolitis), allergy and irritant / toxic health reactions. Most commonly the adverse health reactions are of short duration and reversible, provided the problem has been identified correctly and the exposure has been stopped. In some rare cases the health consequences may be more serious and irreversible.

The papers in this book will provide a deeper and more detailed understanding of these problems. The scientific focus of the conference program and peer-review of submitted presentations was developed and carried out by a program committee of internationally recognized leaders in the field that included Harriet Ammann, Ph.D., Washington State Department of Ecology, Air Quality Program, Olympia, WA, U.S.A.; Pierre Auger, M.D., Direction de santé publique de Québec, Service de l'environnement, Beauport, Québec, Canada; Leila Brickus, Ph.D., Fundacao Osvaldo Cruz, ENSP-CESTEH, Laboratorio de Toxicologia, Indoor Air Quality Program, Rio de Janeiro, Brazil; Prof. Manfred Gareis, D.V.M., Institut für Mikrobiologie und Toxikologie, Bundesanstalt für Fleischforschung, Kulmbach, Germany; Nceba Gqaleni, Ph.D., University of Natal, School of Medical Sciences, Durban, South Africa; Tuula Husman, M.D., National Public Health Institute, Division of Environmental Health, Kuopio, Finland; Laura Kolb, Ph.D., United States Environmental Protection Agency, Washington, DC, U.S.A.; Erwin Martlbauer, D.V.M., University of Munich, Veterinary Department, München, Germany; Claude Mainville, Eng. Naturair Kiwatin, Montreal, Quebec, Canada; Phil Morey, Ph.D., AQS Services Inc. Gettysbury, PA, U.S.A.; Prof. Aino Nevalainen, Ph.D., Kuopio, Finland, National Public Health Institute, Division of Environmental Health, Kuopio, Finland; Ed Olmsted, CIH, Olmsted Environmental Services, Garrison, New York, U.S.A.; Prof. Anna-Liisa Pasanen, Ph.D., University of Kuopio, Division of Environmental Health, Kuopio, Finland; Thomas Rand, Ph.D., Department of Biology, Saint Mary's University, Halifax, Nova Scotia, Canada; Robert Samson, Ph.D., Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands; Linda Stetzenbach, Ph.D., University of Nevada, Las Vegas, Nevada, U.S.A.; Torben Sigsgaard, M.D., Ph.D., Institute of

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The presentations in this book will cover questions related to:

A. Health effects, Pathology

What are the health effects and how do we assess and measure them?

What should be tested to accurately assess exposure?

Who are the susceptible persons or groups?

What new epidemiology and case studies have been performed; what regional differences in exposure and effect occur, - including studies from countries in transition.

B. Exposure Assessment and Analytical Methods

Why is there exposure and how do we assess and measure it?

What are the current developments regarding analytical tools to characterize exposure?

What are important microbial agents?

What is the relationship between moisture and bio-deterioration?

Are there any reliable threshold levels for agents in exposure assessment?

What is the prevalence of moisture defects and bioaerosol problems in countries in transition; - are there regional differences?

C. Application, Prevention, Remediation and Control

What is the relationship of building methods/materials and bioaerosol production?

How can indoor contamination be managed?

When should a building be evacuated?

What are the differences between traditional and modern building architecture; - what comparison of bioaerosol contamination risk can be made?

What are modern remediation technology and clearance criteria for building materials and furnishings?

Are there significant case studies relating to building codes and bio-deterioration, public health experience and risk assessment?

What experience do we have with “threshold limits”?

D. Clinical Case Studies

What medical case studies regarding allergy, infection, irritant and toxic effects, intervention studies exist?

Are there innovative diagnostic and therapeutic approaches?

What is the psycho-social impact on patients of indoor air problems from microbial contamination?

I wish to thank all of the committee members for their extraordinary dedication, time and work. I want to give a special note of thanks to the reviewers for their professional contributions, and most important to the presenters and participants for their great efforts and time to make this again a unique and successful meeting. May this book and the papers from a wide variety of investigators contribute to the advance of the scientific knowledge and assist in public health initiatives to protect building occupants and workers and the that end, prevent unnecessary suffering and costly diseases caused by microbial exposures.

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

Conference Chair

Fungal Research Group Foundation,
Albany, New York.

INTRODUCTION

Ladies and Gentlemen, Dear colleagues,

I wish to extend to you a warm welcome to the 5th International Scientific Conference on Bioaerosols in Indoor and Work Environments!

Since our first meeting in 1994 here in Saratoga Springs, New York, we have met here in 1995, 1998 and the last time with our friends in Helsinki, Finland in 2000 as part of the Healthy Buildings meeting. I think we can all agree that there has been additional work and evidence linking indoor and occupational microbial exposure from contamination with fungi, bacteria and their by-products to diseases with important public health implications, such as allergy, asthma, inflammatory lung diseases and airway infections. There have been more studies of inhalation-related mycotoxicosis and its manifestation in humans. Many of the experts now believe that probably several biological agents, rather than one single substance, occur in “real-life” situations - some substitute it with “moisture or water damage” - and are responsible for the various adverse human health reactions. The environmental testing, medical diagnosis and appropriate treatment of some patients not seldomly creates challenges for field investigators, clinicians and public health officials. Climate changes, air tight buildings, envelopes with water infiltration and condensation problems, porous materials, inadequate building and ventilation design and maintenance, transfer of building materials and designs to countries with different climates and economical resources, and other factors appear to contribute to microbial exposure.

A remarkable growing international concern about microbial exposure on the building occupants' health and actions by public health professionals are notable. Responsible administrators and building managers are now addressing “moldy and wet” schools, libraries, nursing homes and apartments. “Mold is here to stay” - There is hardly any professional indoor air conference over the last several years that does not cover the topic of “mold and bacteria” in some form or another. Nevertheless, we the organizers, believe that this conference is unique and special - it meets a need that is not addressed anywhere else in this form and content - that is why some of you have been “regulars” and active participants in the presentation of new discoveries, research, discussions and scientific debate and we attract new participants from around the world.

The conference will address the state of the art in the science and application of recent research and practical experience to better assess microbial exposure, to

determine important agents and adverse human health effects and to recommend treatment, control and prevention guidelines for fungal and bacterial exposure. One focus will be to connect the experience of different countries, of leading researchers in the field and of various “front line” practitioners and consultants in the “real-world”. From more than 120 papers representing twelve countries around the world, we have selected through critical peer-review, 70 papers of original and new research and findings that will be presented by the authors. There will be sufficient time to reflect on the work and exchange comments and ideas. Great emphasis will again be placed on practical questions (remediation and prevention) as well as on the professional exchange of “real-life” questions, patients’ problems and technical approaches.

I hope you have a great time at the conference meeting and hope that you can catch up with your old friends and colleagues and that you can meet some new ones as well - besides perhaps finding time to enjoy the foliage colors in the North Country,

in the name of the Organizing committee,

Eckardt Johanning

Chair of the Scientific Committee

Acknowledgment

I would like to thank all scientific committee members for their extraordinary dedication, time and work and a special note of thanks to the reviewers for their professional contribution.

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ENDORSEMENT & SUPPORT



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University of Natal (South Africa) - Centre for
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Gesellschaft für Mykotoxin-Forschung (Mycotoxin Research Association, Germany)

International Commission Occupational Health
– Organic Dust Workgroup

MANUSCRIPTS

Chapter 1

Health Effects I – Epidemiological Research

Session chairs:

Torben Sigsgaard, Carl-Gustav Bornehag

WHAT DO WE KNOW ABOUT DAMPNES IN BUILDINGS AND HEALTH? CONCLUSIONS FROM TWO MULTIDISCIPLINARY REVIEWS OF THE ENTIRE LITERATURE ON DAMPNES IN BUILDINGS AND ASSOCIATED HEALTH EFFECT

Torben Sigsgaard¹ and Carl-Gustaf Bornehag²

¹ Department of Environmental and Occupational Medicine, Aarhus University, Denmark

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ABSTRACT

The scientific literature on dampness in buildings and health has been reviewed by a Nordic and a European expert group (NORDDAMP and EUROEXPO) and up-dated by the authors. The major conclusion is that there is strong evidence for that moisture related problems in buildings increase the risk for health effects among atopics and non-atopics and children and adults both in domestic and public environments. The most important health effects that are associated to dampness in buildings seem to be airway symptoms such as wheezing, cough and asthma. Furthermore, general symptoms and airway infections seem also to be associated to dampness indoors. Relative risks for living or working in damp buildings are in the range of OR 1.4-2.2. However, the literature is not conclusive in respect of causative agents in such buildings. Suggested causative agents are mites, microbiological agents and organic chemicals from degraded building materials. A general conclusion from the works is that there is a strong need for more multidisciplinary studies including competence from all relevant areas. There is also a strong need for multidisciplinary reviews in scientific journals on articles including associations between indoor environmental factors and health effects. Finally, the recommendation to the general public is to remediate damp buildings and to avoid mite exposure.

INDEX TERMS: Adverse effects, Dampness, Molds, Review, Allergy

BACKGROUND

Several epidemiological studies have reported an association between “damp” building and health problems such as asthma and other respiratory symptoms.

Furthermore, some studies suggest that living in “damp homes” is a risk factor for sensitisation and development of allergy (Commission on Environmental Health, 1996; Sundell and Kjellman, 1994). However “dampness” is a rather vague concept, with variable definitions, and specific chemical or biological factor that can explain the association needs to be identified. Some issues that need to be evaluated are:

- What health effects are associated with dampness in buildings?
- Is the shown associations caused by bias?
- Which are the health relevant moisture related exposures indoor?
- What is a health relevant definition of a damp building?

METHOD

One Nordic and one European expert group have reviewed the entire literature on dampness in buildings and health. The Nordic group (NORDDAMP) reviewed the literature up to July 1998 (Bornehag *et al.* 2001). The European group (EUROEX-PO) included the literature from August 1998 to 2000, (Bornehag *et al.* 2004). A description of the review method is described in the first of these reviews. Furthermore, epidemiological studies with objective evaluation of both environment and health, published after the before mentioned periods have been reviewed by the authors and are described in more detail in a special section of this report.

The included articles were discussed and evaluated in the two groups on the basis of the following criteria:

- Is exposure and health effects charted and described in a relevant manner, with respect to e.g. time of exposure (e.g. infancy, childhood years, and actual exposure)?
- Is the selection of the investigated persons satisfactory?
- Is the statistical analysis including control of “confounders” and effect modification satisfactory?

RESULTS

In total, 1137 articles were identified in the two reviews. Of these, 920 were excluded - by reading abstract - as they were deemed as not relevant for the question at issue. Consequently, 217 articles were reviewed by the two groups and of these 116 were excluded as they were judged to be background papers, “non-informative” or “inconclusive”. The term “non-informative” indicates that the article lacks essential information concerning exposure or health effects or that the analysis did not consider possible confounding from other factors. The term “inconclusive” indicates that data processing or reporting makes it impossible to draw conclusions

concerning any relationship between exposure to “dampness” and health effects. It should be pointed out that many of the excluded studies have had other objectives than to investigate associations between “dampness” and health. Consequently, 101 articles were judged as relevant and conclusive and has been the foundation for this work.

In the following presentation the studies have been divided into one or more of 4 categories depending on the type of data for exposure:

- Self reported dampness and self reported symptoms
- Self reported dampness and clinical findings incl. physician diagnostic
- Observed dampness (i.e. inspections and/or measurements) and self reported symptoms
- Observed dampness and clinical findings

Studies on self reported dampness and self reported symptoms

This category included the largest number of studies, in total. 66 studies. The design of these studies was mainly retrospective cross sectional investigations and case-control studies. The group of studies showed a consistent association between dampness and health effects. Associated health outcomes were mainly airway symptoms such as wheezing, cough and asthma.

Studies on self reported dampness and clinical findings

20 studies were identified in this group. Of the 14 studies in NORDDAMP, 6 investigations reported significant positive associations. In EUROEXPO, five of six studies showed significant associations. Self reported dampness was mainly associated to doctor diagnosed asthma and the severity of asthma. In a few studies there was reported an association between dampness and sensitisation.

Studies on observed dampness and self reported symptoms

In this category, 28 studies were judged, 23 investigations in NORDDAMP and 5 studies in EUROEXPO. In 8 studies including data from inspections, there were significant associations to symptoms. Furthermore, in two of these studies the association between inspected dampness and symptoms was stronger than between self reported dampness and symptoms. Other studies in this group mainly included exposure measurements and its association to symptoms. In this group significant associations were reported, however, the findings are not consistent.

Studies on observed dampness and clinical findings

Thirteen studies with objective data concerning exposures (inspections and exposure measurements) and clinical findings were identified. In ten of these investigations significant associations were reported mainly between different kinds of microbiological exposures and airway diseases. However, the review groups did not find any consistent results.

RECENT STUDIES

In a study from Finland the authors followed the changes in inflammatory markers in nasal lavage (NAL) and induced sputum before and after summer vacation and again before and after winter vacation (Purokivi *et al.* 2001). In this study the water damaged schools had slightly but significantly higher amounts of mold spores in the air compared to dry schools, GM were 29 vs. 6 cfu*m⁻³ respectively. The study found significantly higher levels of pro-inflammatory cytokines in NAL from the children from the water damaged schools during the spring term also they could demonstrate a drop in these cytokines during the summer holiday. In induced sputum, this was only found for IL-6.

Wälinder and co-workers studied 12 office workers from water-damaged offices and 8 controls from dry offices. The total number of bacteria were (arithmetic mean) 31,000 and 20,000 and total molds were 140,000 and 87 in the wet and dry offices respectively. These differences corresponded to significant differences in Median (interquartile range) in the two groups of 2.8 (2.1-5.5) vs. 1.4 (1.1-1.6) ECP mg/l, 41.0 (26.8-140) vs. 8.8 (2.5-25.5) MPO mg/l and 12.6 (5.7-18.1) vs. 3.0 (3.0-3.8) Albumin mg/l (Wälinder *et al.* 2000).

Smedbold *et al.* (Smedbold *et al.* 2002) studied Nurses in geriatric hospitals in the middle of Norway. They found that the presence of *A. fumigatus* and higher temperatures were related to a lower cross sectional area and volume. However, acoustic rhinometry was carried out as near as 20 after nasal lavage, and therefore some of the variation might be due to this timing of the procedure. Also the authors did not normalise the nasal volume to the highest volume possible in the same individual as seen i.e. after treatment with a decongestive agent. In this study no effect was found on inflammatory markers in the nasal lavage.

A recent study tried to examine the relation between the actual exposure to mold spores, and the NAL cytokine level. After dividing the material into high and low mold exposure higher concentrations of NAL cytokines were found among the high exposed persons (Roponen *et al.* 2003).

Two studies of childhood allergy related to mold exposure have been published during the last few years. In the first Jacob *et al.* studied indoor mold exposure in allergic children of three areas of Germany, where studies on ambient air and respiratory symptoms were ongoing. In a nested case control manner 115 sensitised children were compared to 157 non-sensitised non-symptomatic children 5-10 years of age. The authors found that any sensitisation towards common allergens was increased in children exposed to high levels (> 90 percentile) of *Aspergillus* and *Cladosporium* whereas *Penicillium* was a weaker risk factor for sensitisation. These associations were not affected by controlling for house dust mite (*Der p1* and *Der f1*) exposure in the model. Furthermore, it was shown, that the exposure to mold spores increased allergic symptoms in all children independently of sensitisation.

In a study of high-risk children from the Boston area it was shown, that the incidence of asthma increased with the presence of molds in the home. When this was further broken down to species this increase in risk was shown to be related to the occurrence of *Zygomycetes spp.*, *Cladosporium spp.* and *Alternaria spp.* in the bedrooms of the children (Stark *et al.* 2003).

DISCUSSION

The major conclusion is that there is strong evidence for that moisture related problems in buildings increase the risk for health effects among atopics and non-atopics and children and adults both in domestic and public environments.

What health effects are associated with dampness in buildings?

The most important health effects that are associated to dampness in buildings seem to be airway symptoms such as wheezing, cough and asthma. Furthermore, general symptoms and airway infections seem also to be associated to dampness indoors. Relative risks for living or working in damp buildings are in the range of OR 1.4-2.2. For allergic sensitisation the recent German study emphasises the potential adjuvant effect on sensitisation by molds.

Is the shown associations caused by bias?

In many countries around the world, there is a broad opinion that moisture and mold problems indoor is a risk factor for asthma and allergy. This, in combination with dependent collection of data as in questionnaire studies, i.e. persons reporting both exposures (dampness) and health effects (symptoms), increases the risk of reporting bias. Symptomatic people may be more concerned about moisture problems and/or persons living/working in buildings with moisture problems may be more observant on symptoms, and thus more inclined to report symptoms. This could partly explain the reported associations. However, it was shown that studies

including independent measurement of exposures (i.e. inspections) and health effects (self reported symptoms and/or doctor diagnosed diseases), showed even stronger association between “dampness” and health effects compared with results from questionnaire studies. Therefore, selection bias cannot explain all the reported associations.

Which are the health relevant moisture related exposures indoor?

There are findings showing associations between mainly microbiological exposures indoor and health effects such as asthma and allergy. However, the literature is not conclusive in respect of health relevant agents in buildings with moisture problems. Suggested causative agents are mites, microbiological agents and organic chemicals from degraded building materials.

Mites: Exposure to mites has been shown to increase the risk of sensitization and allergic disease. In all studies where adjustment for mite exposure has been made, the association between dampness and health decreased, but still remained. The OR of the association between dampness and health seems to be almost the same all over the world, with very varying climate conditions and different prevalence of mite infestation. So, mite exposure cannot explain all health effects related to damp buildings.

Microbiological agents: The possible role of microbes/molds as a (major) causative agent for dampness related health effects is unclear, and the literature is still not conclusive, although the more recent studies seem to point towards a general inflammatory effect on the mucous membrane. The prevalence of sensitisation to mold allergen is low; however, mold exposure seems to be a risk factor for sensitisation per se and there is a possibility of yet unrecognised fungal components, like i.e. the fragments of hyphae that has been shown to be liberated from some species during growth (Kildeso *et al.* 2003).

Organic chemicals: High humidity content in materials can give rise to degradation procedures, with emission of substances as a result. Such compounds can give rise to odour, irritation and maybe allergic reactions. However, the literature concerning dampness-related health effects from such compounds in relevant concentrations is missing.

What is a health relevant definition of a damp building?

The conception of “dampness” includes both humidity in indoor air and moisture in the construction of which both have been associated with health problems. There are data indicating that different part of the world, at least partly, have different kinds of “dampness” problems. In e.g. Scandinavia visible mold and con-

condensation on walls seems to be rather rare while humidity in the construction with indications such as bad odour seems to be more frequent. In more humid climates as in e.g. Taiwan the frequency of e.g. visible mold and condensation on walls are much higher. However, the reported risks for health effects are in the same range regardless of the prevalence of such “dampness” indicators, which is remarkable. In conclusion, it is not possible to give a more precise health relevant definition of a “damp” building.

CONCLUSIONS

The review have shown strong evidence for that moisture related problems in buildings increase the risk for health effects airway problems such as asthma and allergy. However, the causal relationship in such buildings has not been identified yet. The review has also shown that there is a strong need for multidisciplinary reviews in scientific journals of articles including associations between indoor environmental factors and health effects. It is essential to carry out more really multidisciplinary studies, including competence from all relevant areas. The studies should not only have a cross-sectional design, but also be of longitudinal prospective type as well as intervention studies. The recommendation to the general public is to remediate damp buildings and to avoid mite exposure.

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MOLD REMEDIATION OF A SCHOOL AND TEACHERS' HEALTH

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ABSTRACT

Effects of indoor mold exposure on teachers' health were studied before and after mold remediation in a school center. Health data was collected three times with questionnaires from teachers (n=31) working in mold damaged school building and from a reference group (n=13). Spirometry was measured three times. IgG antibodies to 20 different microbes were determined twice by enzyme-linked immunosorbent assay (ELISA). In the study group, the number of sinusitis episodes was higher (p=0.040) and the mean duration of sick leaves was longer (p=0.015) than in the reference group. Eight asthma cases were identified. After the remediation, bronchitis, conjunctivitis and symptoms of allergic rhinitis decreased and no new asthma cases appeared. No changes in the lung function and no differences in 20 mold-specific IgG-antibodies were observed between the groups.

Conclusion: The remediation of the mold damage had beneficial health effects.

INDEX TERMS: indoor air, moisture damage, adults, IgG, spirometry

INTRODUCTION

Mold in indoor environment connected to adverse health effects has aroused public awareness and attention during last decades. Connection between exposure to indoor mold and health effects like respiratory, irritative, allergic and general symp-

toms has been found as well at homes (Peat, 1998) as in urban working places (Sigsgaard, 1999).

Preliminary reports on intervention type of studies have suggested that repair measures decrease the symptoms of the occupants (Åhman, 2000). However, in spite of strong epidemiological evidence about the association between building moisture damage and adverse health effects, little is known of long term health effects of prolonged microbiological exposure.

In this study we followed the remediation process of school building complex and monitored the health of the teachers before and after the remediation with a symptom questionnaire and clinical measurements including mold specific immunoglobulin G antibodies and lung function.

METHODS

School buildings and microbial sampling: The school center under study is located in a small town in central Finland. In the spring 1996, the three buildings of the school center were first visually inspected for the signs of mold growth, moisture measurements and structural openings. Exposure assessment was continued with microbial sampling of indoor air, surfaces and damaged building materials. Based on those investigations, two of the school buildings were considered as moisture-damaged (index) and one of the school buildings as a non-damaged (reference) building.

Indoor air microbes were collected with a six-stage impactor (Andersen 10-800) (Andersen, 1958) from classrooms, halls and personnel rooms during school days (sampling time 10 min). Samples for fungi were taken on 2% malt extract agar (MEA) and dichloran 18% glycerol agar (DG18) and samples for bacteria on tryptone glucose yeast agar (TGY). Sampling campaigns were carried out before and after the intervention, and as a three-year follow-up. Indoor/outdoor ratios (I/O-ratio) of fungal concentrations were used to evaluate differences between the three campaigns. Fungi were incubated for 7 days at 22°C and then identified morphologically mainly to genus. Bacteria were incubated for up to 14 days at 25°C.

Symptom questionnaire study: Health data was collected with self-administered questionnaire from the teachers (n=44) working in the school center three times. The questionnaire was based on previously published sets of questions (Andersson, 1998; Susitaival, 1996) included 70 questions on general and irritation symptoms, respiratory infections, allergy and medication. The first questionnaire was sent before the technical investigations were started in spring 1996, the second when repair were completed (spring 1997) and the third as a follow-up, two years later (spring 1999).

Determination of serum Immunoglobulin G antibodies: Serum samples for the mold-specific immunoglobulin G (IgG) antibody measurements were available for 26 (60%) teachers, of whom 19 worked in the index school and 7 in the reference school. The samples were drawn twice, first at the completion of the remediation in spring 1997 and again in the spring 1999. They were stored in the freezer (-20° C) until analyzed.

Serum IgG antibodies to 20 different microbes were determined by enzyme-linked immunosorbent assay (ELISA). The microbes in were *Aspergillus (A.) fumigatus*, *A. umbrosus*, *A. versicolor*, *Aureobasidium pullulans*, *Cephalosporium curtipes*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Fusarium avenaceum*, *Geotrichum candidum*, *Paecilomyces variotii*, *Penicillium brevicompactum*, *Phialophora bubakii*, *Phoma macrostoma*, *Rhizopus nigricans*, *Rhodotorula glutinis*, *Sporobolomyces salmonicolor*, *Stachybotrys atra*, *Streptomyces albus*, *Trichoderma viride* and *Tritirachium roseum*. The microbes were selected to represent internationally recognized fungi indicative to moisture (Flannigan, 1996) and other microfungi common in Finnish buildings (Hyvärinen, 1993).

Spirometry measurements: Spirometry measurements were started in the spring 1997 and the follow-up measurements were made in the spring 1999 and 2000. A complete series of the three measurements was obtained from 23 (52%) teachers, of whom 17 were from the index group and 6 from the reference group. The same spirometer equipment was used every time. Spirometry was performed with a computerised flow volume spirometer (M905 Medikro Oy, Kuopio, Finland). At least three acceptable forced maximal expirations were performed according to the standards of the American Thoracic Society (American Thoracic Society, 1987). From the maximum expiratory flow volume curves the highest forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and flow rates at highest forced vital capacity (MEF 50, MEF 25) were read. All values were expressed as percentages of reference values in Finland (Viljanen, 1982). The same experienced nurse did all the measurements.

Statistic: Mann-Whitely-U test was used to test the differences between indoor-outdoor (I/O)-ratio of the microbial concentrations. The differences in the occurrence of the dichotomous symptoms and infection variables between the index and reference groups were tested with chi-square test and Fisher's exact test. Wilcoxon's test and Mann Whitley-U test were used to compare absorbance values of mold-specific IgG values between the index group and reference groups (SPSS, 1998). A linear regression for repeated measurements and the MIXED procedure were used to examine the levels of lung function measurements at the end of the remediation and two years later (SAS, 1996).

RESULTS

Microbial results: The airborne concentrations of fungi varied from 4-375 cfu/m³ and in materials between below detection limit (<45 cfu/g) and 22 000 cfu/g at the beginning of the study. The indoor-outdoor-ratio (I/O-ratio) of the fungal concentrations was significantly higher ($p=0.005$) in the index school (1.46) than in the control school (0.33). I/O-ratio of viable fungi in the index school after remediation reverted to the same level with the control school (0.70-0.86), and I/O-ratio of two most common fungi *Penicillium* and *Cladosporium* decreased.

Symptom questionnaire: There were no differences between gender, mean age, smoking, pets, moisture problems at school, years at school and regular medication between the index group and references at the beginning of the study. Altogether 19 (70%) of the index school teachers and 7 (58%) of the references were females, mean age was 47 years and working years at school 17 in the index group and 45 and 15 in the references, respectively.

Self-reported respiratory symptoms, infections and symptoms of allergy due to poor indoor quality at the beginning of the study among the index group and references are shown in Table 1.

In the beginning of the study, hoarseness was more common among the index than reference group ($p=0.04$). Sinusitis episodes were more prevalent in the index school. The mean duration of sick leaves was longer in the index than the reference group; 22 days in the index school vs. 2.3 in the reference school ($p=0.02$). Eight doctor diagnosed asthma cases were found in the index school, while there was none in the reference school. The index group had significantly more complaints about the quality of indoor air; mold odour, cellar like odour, unpleasant odours, stuffy air and dust.

After the remediation, the incidence of self-reported bronchitis and conjunctivitis decreased in the index group and a similar trend was observed in the occurrence of sinusitis. Symptoms of allergic rhinitis were less frequent in the one-year follow-up. No new asthma cases appeared. Among the reference group, there were no changes in self-reported infections and allergic symptoms (data not shown). The complaints of dust or dirt inside the rooms decreased among the index group but the complaints of odour remained the same (data not shown).

Table 1. Health status among the index and reference school teachers at the beginning of the study.

	Index school teachers		Reference school teachers		p
	n	(%)	n	(%)	
1. Self-reported respiratory symptoms					
Fever	7	(26)	4	(33)	0.64
Cough with phlegm	8	(31)	3	(25)	0.72
Wheezing	6	(26)	-		0.06
Dyspnea	10	(40)	1	(10)	0.08
Nasal bleeding	7	(26)	4	(33)	0.63
Rhinitis	20	(77)	10	(83)	0.65
Sore throat	20	(74)	9	(75)	0.95
Hoarseness	25	(93)	8	(67)	0.04
2. Self-reported infections.					
Sinusitis	7	(26)	4	(33)	0.64
Bronchitis	5	(19)	2	(17)	0.89
Conjunctivitis	11	(44)	7	(58)	0.41
Sick leaves	7	(27)	3	(25)	0.90
Mean length of sick leaves, days	22		2.3		0.02
Episodes of sinusitis (mean)	3.3		1.3		0.04
3. Self-reported allergy symptoms					
Allergic rhinitis	19	(70)	7	(59)	0.46
Astma	8	(31)	-		0.04
Atopic eczema	5	(19)	3	(25)	0.64

Mold-specific immunoglobulin G-antibodies: In the IgG antibody concentrations after the remediation, no statistical differences were found between the groups. In the two-year follow-up, the IgG antibody concentration for *Tritirachium roseum* was significantly lower in the index group than at the beginning of the study but no changes in the reference group was observed.

Spirometry measurements: In the lung function measurements after the remediation no differences between the groups nor changes in lung function follow-up were observed (data not shown).

DISCUSSION

This study was conducted in a school center because of long-term mold problem of school buildings and health complaints of the personnel. According to structural and microbiological investigations before the remediation the exposure measured by microbiological and building-physical methods was higher in index school and after the remediation it was diminished to the level of reference school building.

At the beginning of the study, the prevalence of asthma was high. Increased risk of asthma has been reported earlier in association with moisture and mold exposure (Peat, 1998). The symptom rates of the index school teachers were increased as well as subjective annoyance by many indoor physical factors. The number of sinusitis episodes was higher than among the reference group, which has also been observed in earlier studies (Koskinen, 1999). The mean length of sick leaves was remarkably higher in index group than in the reference group at the beginning of the study. This is a new finding with respect to adults exposed to indoor mold. A similar finding was observed in our previous study in children attending to moisture-damaged day-care center (Koskinen, 1999).

In the follow-up no new asthma cases appeared but asthmatic symptoms were equally frequent. A slow recovery among mold exposed teachers has also been reported earlier (Rudblad, 2002). A decrease of respiratory infections was found, and reports of perceived poor indoor quality had decreased. Indoor mold exposure has been connected to a variety of infections in airways (Peat, 1998). Thus, the decrease in the infections after the elimination of mold exposure speaks for a causal connection between exposure and respiratory infections, although the etiological aspects remain to be clarified.

Overall concentrations of IgG antibodies to 20 molds in the index and reference teachers did not differ. Similar findings have been reported in schoolchildren (Immonen, 2002). These microbes can be found also in other environments, outdoors and other buildings. According to the results of this study the IgG levels do not reflect recent or specific exposure to indoor molds.

In spite of high number of clinically verified asthma cases, there were no significant differences in the mean values of lung function measurements between the index and reference group and during the follow-up study. In this study, mold exposure in the working environment did not lead to measurable changes in the lung function in spite of high frequency of symptoms.

CONCLUSIONS

Moisture and mold damaged school environment may be a risk factor for such severe health outcomes like respiratory infections and asthma. According to follow-up study, the remediation of the moisture damage had a beneficial effect of the exposed personnel.

ACKNOWLEDGMENTS

The authors thank Mrs Kirsti Nilsson, specialist nurse, for taking all the blood samples and performing all the spirometry measurements of the teachers. We also wish to thank Mr Pekka Tiittanen, MSc for his advice in the statistical analyses. We are also grateful to the teachers and town administration for making this research possible. The study was financially supported by the Finnish Research Programme on Environmental Health, Academy of Finland, Yrjö Jahnessons Foundation and The Finnish Work Environment Fund.

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ASSESSMENT OF EXPOSURE TO INDOOR ENVIRONMENTAL FACTORS FOR AN INFANT COHORT AT RISK FOR ASTHMA: PRELIMINARY ANALYSIS OF BIOAEROSOL DATA

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ABSTRACT

Environmental risk factors for asthma development are being assessed in a birth cohort of >100 infants of asthmatic mothers. The overall hypothesis is that relationships exist between environmental factors and the infant's respiratory health. Objectives include assessment of bioaerosols and other environmental factors within infant's homes, and the development of a non-standard protocol for bioaerosol collection. Measurements included bioaerosols, gravimetric particulate matter, infant cord blood cotinine and urine cotinine, indicators of environmental tobacco smoke (ETS) exposure. Home survey and medical history data were collected. Preliminary, partial results are presented. Smoking was strongly associated with ETS exposure indicators. The median indoor CFU/m³ was 1162 in summer, and 327 in winter; median indoor/outdoor ratios were 0.8 in summer and 1.8 in winter. To date, 186 taxa of fungi have been identified. Isolates of *Aspergillus sp.* were prevalent in the initial home visits of the 38 homes of infants with medically-diagnosed wheeze.

INDEX TERMS: bioaerosols, indoor environmental quality, environmental tobacco smoke, infant wheezing, asthma

INTRODUCTION

From 1980-1993, the prevalence of asthma in the U.S. increased by 75% with the largest increase occurring in children under 5 years of age (CDC, 1994). Identified risk factors for asthma include: excessive allergen and tobacco smoke (Utell, Looney, 1995); gas stove exposure and nitrogen dioxide (Ciuk *et al.* 2001); and exposure to fungi such as *Penicillium spp.* (Licorish *et al.* 1985), *Aspergillus spp.* (Garrett *et al.* 1998), and *Alternaria spp.* (Negrini *et al.* 2000). The potential for multiple agents to be involved with asthma has led investigators to assess such exposures in the indoor environment (Samet, 1995). Although links have been established between fungi and asthma (Stark *et al.* 2003), there is a need to improve protocols to isolate and identify under-represented slow growing fungi from air samples. The long-term application of this study is targeted on the development of primary intervention strategies to reduce disease severity.

The homes recruited for this study were selected from a pool of residences identified in Syracuse, as part of the *Assessment of Urban Dwellings for Indoor Toxics* program. The primary recruitment criterion was the pregnant woman with a history of asthma. Three objectives were identified: 1) compilation of an assessment of bioaerosols (fungi, bacteria) in study homes; 2) development of improved protocols for collection and isolation of fungi; and 3) determination of relationships, if any, among environmental factors, including fungal species and other parameters. In addition to field measurements, medical histories and home surveys were completed. Although 153 homes were studied from December 2001 until March 2003, much of this report presents an analysis of selected factors of 2002 data.

METHODS

During 2002, 130 homes were sampled, 103 initial collections, representing an independent data set analyzed for this report, and 27 seasonal repeat home visits. Homes with missing values were excluded from the analysis. Methods were described in standard operating protocols, reviewed by the institutional review board (IRB), SUNY Upstate Medical University, and incorporated into a quality assurance project plan (QAPP) approved by the US EPA and the New York Indoor Environmental Quality Center.

Measurements:

- A Integrated collection over a 20-24 hour period of discrete samples for:
- 1) total viable fungi and bacteria, CFU/m³, calculated from actual plate counts (Andersen N6 Sampler), identification determined from microscopic examination;

- 2) particulate matter less than 10 micrometers, PM10 and less than 2.5 micrometers, PM2.5 (Harvard Impactor, gravimetric analysis following USEPA IP10-A); and
 - 3) nitrogen dioxide (NO₂) collected over 7 days (Radiello® passive diffusion monitor; analysis by ion chromatography);
- B Continuous monitoring with samples taken every minute over a 20-24 hour period by real time sampler (Langan, Model L76) for carbon dioxide (CO₂), air temperature and relative humidity;
- C one-time survey data of visible water damage, visible mold, pets, air conditioning; and;
- D medical histories comprising an initial examination at birth including cord-blood cotinine, and quarterly examinations up to one year including measurements of urine cotinine, although only the first 2 quarters are included here. Preliminary analysis of medically-diagnosed wheeze in the first year of life is also included.

Sampling method for fungi: An improved protocol was developed for the isolation and identification of viable fungi and bacteria. Viable microorganisms were impacted for 3 or 6 minutes onto 3% malt extract agar (MEA) plates used for indoor and outdoor samples taken at the beginning and end of a 24-hour period. There were three indoor sampling periods, two for day 1 and one for day 2. One outdoor sample was taken on each day.

For each sampling period two plates were obtained. The un-sampled plate (plate 1) was left unopened after impaction. Following the typical analysis procedures for Andersen plates, colonies were counted at 2 and 7 days after sample collection. For the sampled plate (plate 2), 50 of the 400 impaction points were selected utilizing random number-generated templates, and transferred to 5 fresh MEA plates within 3 hours after collection. Each of these 50 randomly selected “plugs” was transferred aseptically to five “random 50” (R50) plates. On the sampled plate, 350 impaction points were left for counting. Colony forming units, (CFUs), were counted at 2 and 7 days after collection on both the “sampled” and “R50” plates. Fungi present on R50 plates were further studied and categorized into groups based upon similarity or taxon.

Statistical analysis of survey variables: The Shapiro-Wilk test indicated that none of the quantitative variables followed a normal distribution. Natural logarithm was used to transform quantitative variables. Data from home surveys were usually encoded as present or absent. Two-sample t-tests (95% confidence, $\alpha = 0.05$) were conducted for survey variables with two categories and ANOVA was performed on variables with greater than two categories. The least significant dif-

ference test (LSD) was used for mean comparison ($\alpha=0.05$) of log-transformed variables.

Analysis of CFU/m³ indicated that there was no significant difference between the results of samples with 3 minute duration versus those at 6 minute duration, so these data were combined. ANOVA was conducted to test the differences in CFU/m³ among the three sampling types, un-sampled, sampled, and R50. Pearson correlation coefficients were computed between the CFU/m³ results within a given sampling method (e.g., un-sampled) and among the three sampling methods.

RESULTS

Bioaerosols: Mean concentration of viable CFU/m³ in air samples indicated significant seasonal fluctuation (Table 1) similar to that of Shelton *et al.* (2002). The median ratio of indoor/outdoor CFU/m³ of winter samples (1.8) was 2.25 times that of summer samples (0.8), in contrast to Shelton *et al.* (2002).

Table 1. Fungal concentrations and ratios by season (103 homes, 2002).

CFU/m ³	Indoor Summer	Indoor Winter	In/Out Summer	In/Out Winter
Median	1163	327	0.8	1.8
Mean	1626	721	0.9	9.3
Range	172-5880	47-4642	0.1-2.1	0.2 -139.5

The Ward clustering method classified homes into five groups (Table 2) according to inside CFU/m³ for D1T1 (day1 time1) for un-sampled plates. Discriminant analysis detected significant differences (no overlap) among assigned clusters. Similarly, five distinct groups were also observed for inside CFU/m³, D1T1 of R50 plates.

Table 2. Summary of discriminant analysis results for home clusters, CFU/m³ data for 2002. Significant differences existed between all groups (p-value<0.0001).

Cluster	Indoor CFU/m ³ D1T1 (un-sampled plates)				
	n ¹	Mean	Median	Minimum	Maximum
Very Low	50	252	196	47	511
Low	17	739	714	583	1011
Moderate	16	1327	1303	1071	1607
High	7	2192	2178	1821	2595
Very High	8	4075	3839	3380	5880

¹ n: number of homes in each cluster.

To date, 184 taxa, non-sporulating hyaline fungi, non-sporulating dark fungi and “unknown” sporulating hyaline fungi have been identified from 14,552 isolates observed on 11,629 “voucher” slides of viable fungi and bacteria. Identifications are ongoing for 2001 and 2003, partial year sampling. Ten groups comprise 92% of the total isolates (13,601) identified for 2002 (Table 3).

Table 3. Summary of isolates from Syracuse, NY homes 2002.

Microbial groups	Percent of Total Isolates (n=13,601)	Percent of Inside Isolates (n= 8130)	Percent of Outside Isolates (n=5471)
hyaline hyphae, non-sporulating	29	25	34
<i>Cladosporium spp.</i>	19	16	2
Basidiomycetes	8	7	9
<i>Penicillium spp.</i>	8	11	3
Bacteria	8	10	5
<i>Aspergillus spp.</i>	6	9	2
dark hyphae, non-sporulating	6	6	8
yeast	5	5	4
hyaline hyphae, sporulating	2	2	2
<i>Alternaria spp.</i>	1	1	2
Column Totals	92	92	93

Evaluation of non-standard sampling protocol for bioaerosols: Pearson correlation coefficients, ranging from 0.89 to 0.98 for inside CFU/m³ results and from 0.77 to 0.94 for outside CFU/m³ results, showed high linear correlation (p value <0.001) between R50 plates and total (un-sampled) plates. While strong linear relationships exist between sampling methods, ANOVA results indicated that there was significant difference (all p-values<0.0001) between un-sampled and R50 CFU/m³ and between sampled and R50 CFU/m³ but not between un-sampled and sampled CFU/m³. Indoor un-sampled and sampled CFU/m³ results can be estimated (Formulas 1 and 2, respectively), with high confidence, from their R50 results (R²= 0.92 and 0.94, respectively).

$$1. \text{ indoor un-sampled CFU/m}^3 = 22.33 + 0.78 (\text{indoor R50 CFU/m}^3)$$

$$2. \text{ indoor sampled CFU/m}^3 = -6.29 + 0.81 (\text{indoor R50 CFU/m}^3)$$

Pearson correlation coefficients indicated that the correlation between the two day 1 measurements (0.86 to 0.92) was stronger than correlation between day 1 and day 2 measurements (0.59-0.61). While no correlation was observed overall (2002) between CFU/m³ and either PM2.5 or PM10, correlation was observed for data analyzed by season. During summer (June-Aug), inside CFU/m³ D1T1 was negatively correlated with PM2.5 (-0.43, p-value=0.0290) and PM10 (-0.50, p-value=0.0100) indicating possible seasonal effects such as increased ventilation in summer and possibly greater incidence of smoking indoors in winter.

Relationships between survey variables: Significant differences were observed between the 5 categories of home survey observations and 12 environmental and clinical measurements (Table 4). Indoor measurements of CFU/m³ were greater in the presence of carpets, greater with the furnace off than with the furnace on and greater in summer than in winter. In addition to Table 4, no significant differences in CFU/m³ were observed between homes with or without: visible water damage, visible mold inside the home, pets, and air-conditioning.

Analysis of the presence of wheeze during the first year of life is ongoing and at the time of this analysis all infants had yet to attain the age of one year. As an update, medical records indicated wheeze for 38 of the 103 babies that have completed the study. Preliminary examination of fungi found in initial visits of these 38 homes indicates high prevalence of hyaline unknown sp., *Cladosporium sp.*, *Penicillium sp.*, *Aspergillus sp.* and basidiomycete unknowns. Results from 2002 indicated that ninety-one percent of infants with wheeze live in homes with one or more smoker. The identified CFU/m³ clusters in Table 2 (in this sample from 2002) were associated with wheeze detected during the first year of life as follows: Very Low 18/47 (38%), Low 5/16 (31%), Medium 5/15 (33%), High 3/7 (43%) and Very High 3/7 (43%).

DISCUSSION

One of the most important aspects of this study was that data were compiled simultaneously for health parameters and environmental factors at the homes of pre-disposed infants. Season and use of a furnace for all homes were associated with significant differences in CFU/m³, CO₂ and NO₂. The presence of smokers was associated with significantly greater cord blood and urine cotinine consistent with ETS exposure. Both PM_{2.5} and PM₁₀, similarly associated with the presence of smokers, reflect quantitative differences in levels of ETS.

Table 4. Summary of t-test results, p values at $\alpha=0.05$, of 2002 data for five survey variables. “NS” indicates “no significant difference”; “-” analysis not relevant.

	Carpets Present or Absent	Combustion Source Present or Absent	¹ Furnace On (Oct-Mar) or Off	Season (June-Aug) or (Dec-Feb)	Smokers None or At Least One
² PM _{2.5}	0.0103	NS	NS	NS	0.0016
PM ₁₀	0.0148	NS	NS	NS	0.0009
CO ₂	0.0010	NS	0.0081	0.0046	-
NO ₂	0.0365	0.0111	NS	0.0457	-
³ Cot CB	0.0414	-	NS	NS	0.0001
⁴ Cot U3	NS	-	NS	NS	0.0006
Cot U6	NS	-	NS	NS	0.0002
⁵ CFU/m ³ IN D1T1	0.0330	NS	<0.0001	<0.0001	NS
CFU/m ³ IN D1T2	0.0401	NS	<0.0001	0.0003	NS
CFU/m ³ IN D2	0.0402	NS	<0.0001	<0.0001	NS
CFU/m ³ OUT D1	-	-	<0.0001	<0.0001	NS
CFU/m ³ OUT D2	-	-	<0.0001	<0.0001	NS

¹ The “furnace on” period is defined as assumed usage in the Syracuse, NY area

² PM: gravimetric particulate mass measured as $\mu\text{g}/\text{m}^3$

³ “Cot CB”: Cord Blood Cotinine (ng/mL) taken at time of birth

⁴ “Cot U3”: Cotinine measured as cotinine/creatinine ratio ((ng/mL)/mg/dL)

⁵ from infant urine 3 months after birth; “Cot U6” was measured at 6 months after birth

⁶ CFU/m³ were measured on 2 days for “IN” (inside the home), “IN D1T1”, “IN D1T2”, “IN D2”; and “OUT” (outside the home), “OUT D1”, “OUT D2”.

There is no initial evidence from this study that CFU/m³ affects infant health as measured by numbers of infants with wheeze during the first year of life. The association of incidence of wheeze in the first year of life with the identified CFU clusters, Very Low 18/47 (38%), Low 5/16 (31%), Medium 5/15 (33%), High 3/7 (43%) and Very High 3/7 (43%) (Table 2) is confounded by seasonal variation and the analysis has not yet been further reviewed for seasonal effects. Study of the incidence of individual species of fungi in these same homes warrants further review.

CONCLUSIONS AND IMPLICATIONS

The strong association observed between smoking and the ETS markers (cotinine and PM) confirms tobacco smoke exposure and indicates an increased health risk for those infants. Exposure to factors that develop immuno-sensitivity such as species-specific fungal allergens will be quantified when the health surveys have been completed. High percentages of non-sporulating hyaline fungi and Basidiomycetes, a finding unique to this study, indicate that health effects of lesser-known fungi warrant further study and may indicate an increased exposure risk. Although the Andersen sampling method is labor intensive, our R50 sub-sampling method enables the estimation of total CFU/m³ with high confidence as well as more inclusive microbe isolation and more comprehensive identification of these individual isolates.

ACKNOWLEDGMENTS

This research was supported by the Environmental Protection Agency (EPA R-82860501-0), NYIEQ Center, Syracuse, NY and the Syracuse, NY Metropolitan Development Association.

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Chapter 2

Health Effects II – Toxicology and Neurological Effects

Session chairs:

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MOLD TOXICITY: RISK ASSESSMENT FOR HUMANS EXPOSED INDOORS

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Exposure to molds and their products resulting from dampness in indoor environments is not uncommon. Deteriorating housing stock is one source of moisture and resulting microbial growth. In the U.S. a 1995 Housing and Urban Development (HUD) report found that 5.3 million very-low-income renter households (almost 12.5 million individuals) paid more than half their income for housing, much of it substandard and because of disrepair, more prone to suffer from dampness (www.huduser.org/affhsg/worstcase). This number has increased since then, as housing subsidies have decreased in recent years. Dampness problems are not limited to the working poor however, but, as numerous reports in the media have indicated, even new housing for the affluent can have water intrusion problems. Recent work on building materials indicate that some modern building materials may be more prone to microbial growth than was the case for traditional materials (Pasanen *et al.* 1992; Tuomi *et al.* 2000; Murtoniemi *et al.* 2002; Murtoniemi *et al.* 2003 a, b).

Health effects that have been linked to mold exposure include allergy (probably the most prevalent), mucous membrane irritation (and inflammation), neural irritation, adverse responses to odor, and toxicity (Husman, 1996; Ammann, 2002, 2003). This over view concentrates on what is known (and not known) about risk from toxic exposures to molds and mold products in the indoor environment.

In contrast to allergy, from which primarily atopic individuals suffer signs and symptoms, toxicity affects all, but with a fairly wide range of susceptibility to individual and multiple toxins. The amount of exposure may also vary greatly. The effects, as predicted from controlled animal experiments, vary linearly with the amount of exposure.

Mycotoxins, the toxic substances produced by molds, are secreted into the environment on which molds grow, are found on the surface of spores, mycelia and their fragments, and on fine particles generated by molds. They are not thought to be volatile, but some are semivolatile, and some can form an aerosol from surface water/sugar complexes (Jarvis *et al.* 1998). Most toxigenic molds are capable of producing more than one toxin. The toxic potency of such toxins vary by species

and strain of individual mold genera, and range from highly potent (LD50 = < mg/kg; e.g. verrucarins A, J; aflatoxins) to relatively low (100 mg/kg) for others. Toxin production is associated with availability of nutrients, oxygen, water, sporulation and the presence of competitive organisms. Toxin production is thought to convey an advantage for the species or strain against competitors for its environmental niche. Such niches change with changes in building micro-environments, and the succession of species in the microbial communities within buildings. Early colonizers such as *Aspergillus* and *Penicillium* are fast growing, require less water activity in their substrate, and largely disperse their spores passively through the air. Late successional species such as *Stachybotrys* and *Memmoniella* require more water activity and the presence of water for a longer period of time, have a low nitrogen requirement and can grow on cellulose, and disperse their slimy, sticky spores through water or insect transport. However, when dry, such spores can be aerosolized through disturbance, and are of inhalable size (Sorenson, 1995). Most moist environments generally grow a mixture of microbes, including bacteria as well as molds, and the individual molds may produce multiple toxins. Exposed persons could experience additive and synergistic effects from toxins, as well as from other gaseous and particulate materials in indoor air.

Major classes of mycotoxins whose health effects have been studied through *in vitro* or *in vivo* work include (Sorenson, 1993; Reijula, Tuomi, 2003):

- Aflatoxins (*Aspergillus flavus*; *A. parasiticus*)
- Sterigmatocystin (*A. versicolor*, *A. flavus*, *A. nidulans*, *A. rugulosus*, *A. unguis*)
- Trichothecenes (*Fusarium*, *Cephalosporium*, *Trichoderma*, *Stachybotrys*, *Memmoniella*, *Myrothecium* species)
- Fumonisin (*Fusarium verticillioides*)
- Zearalenone (*Fusarium graminearum*)
- Ochratoxin A (*Penicillium verrucosum*, *Aspergillus ochraceus*)
- Ergot alkaloids (*Claviceps* spp.)
- Tremorgens (*Aspergillus* spp.; *Penicillium* spp.).

Systemic effects that have been observed in experimental exposure of animals to various individual toxins, usually from short-term, high level exposures are (Schiefer, 1984; Sorenson, 1993; Coulombe, 1993):

- Immunological (trichothecenes, aflatoxins, ochratoxin A, zearalenone, patulin, citrinin, ergot alkaloids, gliotoxin)
- Hematopoietic (blood-forming) tissues (trichothecenes, aflatoxins)
- Neurotoxic (trichothecenes, tremorgens, ergot alkaloids, aflatoxins, paralytic mycotoxins)
- Hepatotoxic (liver) (aflatoxins, ochratoxins, trichothecenes, fumonisins)
- Nephrotoxic (ochratoxin A, trichothecenes, fumonisins, patulin, citrinin)

- Reproductive (zearalenone, ergot alkaloids, T-2 toxin, aflatoxin)
- Teratogenic (aflatoxin B₁, sterigmatocystin; ochratoxin A, T-2 toxin, zearalenone, rubratoxin)
- Dermatotoxic (trichothecenes)
- Pulmonary toxicity (trichothecenes; aflatoxin sterigmatocystin)
- Carcinogenic (aflatoxins, sterigmatocystins, ochratoxin A, patulin, fumonisins).

It is clear that multiple tissues and physiological systems are affected by individual toxins. Since individual fungi can and do produce multiple toxins, and since in environments, including built ones, toxin-producing fungi usually grow as part of a fungal community comprising multiple species and strains, the entire exposed organism can have multiple effects from exposure.

Determining exposure to individuals living or working in damp indoor environments is highly imperfect. In the recent past, attention has primarily focused on measurement of airborne spores, which is constrained by the complexity of measurement itself. Additionally such attempts are complicated and confounded not only by the biological activity of the fungi as they sporulate episodically, but also by human activity of various sorts, which can disturb and resuspend spores into the air and result in temporal and spatial variations in measurement.

Perhaps even more relevant to attempts to characterize occupant exposure to mycotoxins are recent observations (Gorny *et al.* 2002; Miller, 2004) that toxins are not only found in and on spores, but also on mycelial fragments, and very fine particles that are of microbial origin, or are substrate dust with adsorbed toxins. Miller (2004) found that only 30 percent of recovered mass from settled dust containing toxins was spores; the rest were mycelial fragments (~ 30 %) and very fine particles (~40%). The particulate carriers of toxins may be a much larger source of toxins exposure because of their larger aggregate surface area, and their ability to penetrate deeper into the lung and be transported systemically by various mechanisms.

Attempts to characterize risk from inhalation of spores may give part of the picture of exposure to toxins from the well vascularized nasal epithelium, to the lungs, depending on spore deposition (which is a function of spore size). Exposure of particle-carried toxins has not been measured.

Other attempts to determine exposure (and effect) have looked at biomarkers, antibodies manufactured against toxins to detect toxin presence in bodily fluids or tissues, and “fingerprints” of effect, largely depending on signs and symptoms of effects seen in animal experiments.

Biomarkers for some toxins are well characterized, for instance those for aflatoxin and ochratoxin A are DNA and protein adducts which can be measured in bodily

fluids, and reflect markers of exposure and effect through the products of DNA repair and protein complexing (Sotomayer *et al.* 2003). Metabolites for other mycotoxins can be isolated from urine. Antibodies against mycotoxins have been developed (Vodjani *et al.* 2003 a, b; Fox *et al.* 2004) as have enzyme immuno-assays (Nayak *et al.* 2001).

Risk assessment requires that both toxicity and exposure can be determined. While abundant data exist on short-term or acute effects from high level experimental exposures, relatively few chronic experiments in animals have been done. It may be presumed that in indoor exposures, that except in instances where very high level short-term exposures might occur, such as during uncontained destructive remediation, that the majority of indoor exposures occur over a long time, and at lower levels. This is, of course, an assumption, since investigations of mold exposure usually occur after illness is reported, and exposure has already occurred, perhaps for a long time.

Reconstruction of past exposures is at best problematical. Multiple microbes, bacteria as well as molds, and their products, in addition to non-biological indoor contaminants can influence the disease picture. Microbial exposures can result in allergy and inflammation that can influence susceptibility to toxic agents and influence total effect. Other allergenic agents such as cat, bird or mite antigens, irritant and toxic gaseous and particulate air pollutants and microbial and non-microbial VOCs can elicit concomitant effects. Often more than one toxigenic mold, each of which can produce multiple toxins, may impact occupants. Little is known about additive or synergistic effects of such exposures.

Risk assessment may take into account localized rather than systemic damage (Miller *et al.* 2001). For instance spores or fragments that lodge in a particular portion of the respiratory system may elute their toxins locally, whether this occurs in the nasal passages or in the lower respiratory system. Such considerations might change the dose-response paradigm since relatively few particles, residing over a period of time (or taken up by scavenger cells and transferred to lymph nodes), may deliver a localized, damaging dose. Such point of contact damage has been described through estimation of differential deposition of spores based on size fractionation (Miller, Ammann, 2003).

Host factors of age, state of health, genetic susceptibility, diet, nutrition can influence response to multiple inhalation insults. The complex disease picture that results from multiple exposures that include mycotoxins makes it very difficult to determine what role mycotoxins play in illness. Symptoms are often described as “non-specific” because they are common to several of the exposure agents that have been discussed. Careful differential diagnosis may be able to construct a syndrome that is related to mold/ mycotoxin exposure (Johanning *et al.* 1999). These

include respiratory symptoms such as nasal stuffiness, sinus pain, sore throat, wheezing (not only in atopic patients), chest pain and tightness. Bleeding from the respiratory tract and respiratory distress have been reported (Dearborn *et al.* 2002). Eye irritation is common, as is fatigue. Manifestations of immune modulation such as increased susceptibility to infections that last a long time and are recalcitrant to treatment are often reported (Johanning *et al.* 1996; Gray *et al.* 2003). Nervous system effects such as headaches, memory, attention and cognitive deficits, and sleep disturbance are fairly frequently reported (Gordon *et al.* 1993; Crago *et al.* 2003). Skin lesions and rash also sometimes form part of this picture (Jarvis, 1990; Jarvis, 1995)

Diagnosis is difficult since observed effects are not unique to one toxin but are shared with other toxins and pathogens. Since individual mycotoxins affect more than one system simultaneously, each can produce a multiplicity of effects. Additivity or synergism, which has been demonstrated in the few mycotoxins studied, can play a role. Natural intoxications require information from patients, the patient's environment and other medical history that may influence the disease condition.

Basic science needed for elucidation of mycotoxins' role in disease found in damp indoor spaces include measurement of effects from controlled animal studies of chronic exposures from which dose-response relationships can be determined. Means of measuring exposure to toxins must also be refined since mere measurement of one exposure agent such as airborne spores has not correlated well with symptoms. Development of biomarkers, or markers of effect would help to reduce uncertainty about whether illness seen in damp indoor environments result from exposure to mycotoxins.

In the current state of knowledge, sufficient is known about the degree of toxicity and the potential for exposure to take a prudent public health approach. This includes educating physicians and the public that moisture in building can be controlled sufficiently to prevent problematical microbial growth. When moisture intrusion or condensation from lack of ventilation occurs, the underlying cause of the presence of moisture must be determined, and remediation and clean-up that protects workers and occupants must be carried out.

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CYTOTOXICITY OF DIFFERENT ACTINOMYCETES ISOLATED FROM BUILDING MATERIALS

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ABSTRACT

Microbial damage in buildings often lead to health problems for the occupants. There is strong evidence that toxic secondary metabolites formed by microorganisms play a major role. Complete analysis of all secondary metabolites is not possible, but bioassays can be used to detect cytotoxicity. Previous studies mainly focused on the toxicity of fungi. But Actinomycetes can also be detected in about 60% of moist building materials. We isolated different actinomycetes belonging to the genera *Pseudonocardia*, *Nocardia*, *Nocardiopsis*, *Promicromonospora* and *Streptomyces*. Some strains were tested for cytotoxicity using a modified MTT bioassay with swine kidney target cells. Cytotoxicity varied from no to high toxicity. The most toxic effects were found for *Nocardiopsis sp.* and a strain of *Nocardiopsis exhalans* followed by a strain of *Streptomyces griseus*.

Our results indicate that actinomycetes should not be underestimated in assessing the potential health risk of microbial damages in moisture-laden buildings.

INDEX TERMS: Actinomycetes, Nocardiopsis, cytotoxicity, water damaged building, MTT Test

INTRODUCTION

Residents of water damaged buildings often suffer from health difficulties such as respiratory problems or skin irritations. Rheumatoid problems can also arise (Lorenz *et al.* 2002). How all these difficulties develop has not yet been satisfacto-

rily explained but there is strong evidence that toxic secondary substances from microorganisms can play an important role in this process (Nevalainen, 2002).

In addition to fungi, bacteria are usually found in moisture damaged buildings too. We could show in previous studies, that actinomycetes were present in about 60% of all samples (Lorenz *et al.* 2003).

Up to now, only a few systematic studies have been performed on the occurrence of actinomycetes in moldy houses. There are limited data regarding the species appearing from indoor damage. We have therefore isolated actinomycetes from an air and a dust sample as well as from various moist building materials. As a result, 25 actinomycete strains were isolated from 13 samples. Using morphological characteristics, fatty acid and mycolic acid patterns as well as comparative 16S rRNA analyses, four of the isolates could be identified to species level i.e. *Nocardia carnea*, *Nocardiopsis exhalans*, *Streptomyces griseus* and *Streptomyces violaceoruber*, whereas the other four were assigned to the genera *Promicromonospora sp.*, *Pseudonocardia sp.*, *Nocardiopsis sp.* and *Streptomyces sp.* Based on our results we can conclude that these isolates are strains of new species. Two other isolates still waiting for identification.

It was unclear whether these bacteria produced toxic substances on building materials and thereby led to a potential health risk.

Methods

Eight morphologically distinguishable species were selected for the cytotoxicity tests as well as an additional strain for each of 4 species from another sample or another material: *Nocardia carnea* (2 strains), *Streptomyces violaceoruber*, *Streptomyces griseus* (2 strains), *Streptomyces sp.*, *Nocardiopsis exhalans* (2 strains), *Nocardiopsis sp.* (2 strains of the same new species), *Promicromonospora sp.* and *Pseudonocardia sp.*

The cultures were grown on casein soy peptone (CASO) Petri dishes at about 24°C. After approx. 3 weeks, each culture plate was extracted with 2 x 25 ml chloroform in the stomacher for 4 minutes, the extracts combined, then filtered and subsequently concentrated in the rotary evaporator. The residue was transferred to the sample vial using chloroform, concentrated under a nitrogen atmosphere and subsequently placed in 1 ml cell culture medium. Log 2 dilutions of these samples were then applied to cell culture microtiter plates. Sterile CASO agar culture plates served as control samples and were thus extracted and applied in the same manner.

The cytotoxicity of the samples as well as the control extracts was investigated by means of a modified MTT test with swine kidney target cells as described earlier (Hanelt *et al.* 1994; Gareis, 1995).

Results

The results of the cytotoxicity test are shown in Table 1. The IC₅₀ value indicates the dilution above which the cell cleavage activity is reduced to 50%. Low IC₅₀ values represent a high toxicity and vice versa.

Three of the 12 actinomycete strains examined were found to be highly cytotoxic in the cell culture bioassay: *Nocardioopsis sp.* (strain No. 2a), *Nocardioopsis exhalans* (No. 3b) and *Streptomyces griseus* (No. 4a). Strains No. 2b (*Nocardioopsis sp.*) and No. 4b (*Streptomyces griseus*) only showed a low cytotoxic activity as compared to control R2, while the other strains tested (*Nocardia carnea* (Nos. 1a, 1b), *Nocardioopsis exhalans* (No. 3a), *Streptomyces violaceoruber* (No. 5), *Streptomyces sp.* (No. 6), *Pseudonocardia sp.* (No.7) and *Promicromonospora sp.* (No. 8)) were found to be not cytotoxic.

Table 1. Cytotoxicity of different actinomycetes

No	Strain	Material	IC ₅₀ ¹ [m ² /ml]
1a	<i>Nocardia carnea</i>	wallpaper	>15.62
1b	<i>Nocardia carnea</i>	concrete	>31.25
2a	<i>Nocardioopsis sp.</i>	wallpaper	0.98
2b	<i>Nocardioopsis sp.</i>	plaster	15.62
3a	<i>Nocardioopsis exhalans</i>	loam rendering, paint	31.25
3b	<i>Nocardioopsis exhalans</i>	plaster	3.91
4a	<i>Streptomyces griseus</i>	plaster	7.81
4b	<i>Streptomyces griseus</i>	plastic film, plaster, wallpaper	15.62
5	<i>Streptomyces violaceoruber</i>	dust	>31.25
6	<i>Streptomyces sp.</i>	plaster board, wallpaper	31.25
7	<i>Pseudonocardia sp.</i>	wallpaper, plaster, foam plastic, carpet	>31.25
8	<i>Promicromonospora sp.</i>	wallpaper	>15.62
R1	Sterile plate (reference)	Casoagar	15.62
R2	Sterile plate (reference)	Casoagar	31.25

¹ inhibitory concentration IC₅₀ (minimum concentration which reduces the MTT cleavage activity to 50%)

DISCUSSION

The results of this study show that some actinomycetes, which are commonly found in buildings, can produce cytotoxic substances: certain strains of the genus *Nocardiopsis* (*Nocardiopsis* sp., *Nocardiopsis exhalans*) as well as *Streptomyces* (*Streptomyces griseus*) exhibited cytotoxic activity against the swine kidney target cells used in the MTT test.

Peltola *et al.* (2001) also reported cytotoxic activity with *Nocardiopsis exhalans* as well as other *Nocardiopsis* species and *Streptomyces griseus* in a test using boar spermatozoa as target cells: an inhibition of the spermatozoa mobility and mitochondriotoxic activity was shown. *Nocardiopsis* sp. additionally caused a loss of the spermatozoa membrane integrity.

The toxicity of the *Streptomyces griseus* strains could stem from the formation of Valinomycin, a dodecadepsipeptide, which leads to a loss in mobility of boar spermatozoa which is the result of mitochondriotoxicity of Valinomycin (Andersson *et al.* 1998; Andersson, 1999).

From the strains of the new *Nocardiopsis* species, *Nocardiopsis* sp. strain No. 2a revealed a high cytotoxicity and strain No. 2b only an increased activity. *Nocardiopsis exhalans* strain 3b clearly exhibited cytotoxicity while strain 3a showed none.

Since the strains have not been definitively examined until now, it is possible that the differing ability to produce cytotoxic metabolites is due to varying enzymatic components. Enzymatic differences were also responsible, with *Stachybotrys chartarum* strains for the varying ability in toxin (trichothecene) formation (Peltola, 2001).

In addition, it is known that in the process of subcultivation, the ability to produce toxins can be lost.

Seven of the 12 strains exhibited no cytotoxic effects at all. However, it should be stated that species which were not toxic in pure cultures may produce toxic metabolites in mixed cultures due to the effect of competition for ecological niches.

In addition, the investigation was carried out with agar cultivated cultures.

Since toxin formation is dependent on various environmental parameters (Murtoniemi *et al.* 2001; Puder, 1998), it cannot be ruled out that otherwise harmless bacteria can produce cytotoxic substances on moisture damaged building materials (Hirvonen *et al.* 2001; Roponen *et al.* 2001). This possibility should be examined.

Nocardioopsis dassonvillei is classified in risk group 2 for biological agents (TRBA 466, 2002). Other *Nocardioopsis* strains can cause mycetoma, skin infections, lung infections and conjunctivitis (Kroppenstedt, 1992; Yassin *et al.* 1997). As long as the newly isolated *Nocardioopsis* strains are not fully characterized, the existence of a pathogenic species cannot be excluded.

In the preceding investigations it was shown that building materials contaminated with microorganisms can exhibit cytotoxic activity (Gareis, 1995; Gareis *et al.* 1999; Peltola, 2001; Lorenz *et al.* 2002). The cytotoxicity of the sample materials was often traced back to the fungi (and mycotoxins) found to grow on these materials. The study presented here also point to various actinomycetes which could be responsible for cytotoxic reactions in the cell culture test used.

CONCLUSION AND IMPLICATIONS

Actinomycetes, which appear on moist building materials, can produce cytotoxic metabolites. Therefore, the actinomycetes should not be overlooked when assessing the potential health risk through microorganisms in connection with moisture-related damages in buildings.

The suitability of the MTT bioassay with swine kidney cells as a screening method for the detection of cytotoxic compounds was mainly focused on mycotoxins (Hanelt *et al.* 1994; Gareis *et al.* 2003). However, our results show that this test could also be used for screening the cytotoxicity of bacteria and, with limitations, demonstrate a correlation to the boar spermatozoa test cells.

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SICK BUILDING SYNDROME IN WATER DAMAGED BUILDINGS: GENERALIZATION OF THE CHRONIC BIOTOXIN-ASSOCIATED ILLNESS PARADIGM TO INDOOR TOXIGENIC FUNGI

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INTRODUCTION

There is no academic consensus on whether or not health effects relating to exposure to indoor bioaerosols, sometimes called Sick Building Syndrome (SBS), is a distinct clinical entity (Redd, 2002, Council on Scientific Affairs, 2002). Despite repeated reports of acute and chronic, multi-symptom illnesses acquired by patients following exposure to buildings with both water intrusion and indoor amplification of toxigenic microorganisms (Croft *et al.* 1986; Johanning *et al.* 1996; Hodgson *et al.* 1998; Johanning *et al.* 1999; Sudakin *et al.* 1998; Andersson *et al.* 1997; Dales *et al.* 1999; Dearborn *et al.* 1999; Montana *et al.* 1997; Fung *et al.* 2003: 18; Trout *et al.* 2001), including fungi and bacteria, methodological deficiencies in published studies have precluded drawing definitive conclusions on causation. No studies have identified a robust, objective indicator of neurologic dysfunction to confirm reports of illness. The only medical interventions for SBS to date involved allergy and pulmonary medications or to suggest removal from exposure. The lack of any treatment that could correct the symptoms ascribed to exposure to bioaerosols from water damaged buildings (WDB) has further weakened the contention that SBS is a recognizable illness. Our initial report (43) and the current study addressed the methodological limitations present in the previous studies.

Based on pilot data from 103 cases from 43 WDB (Hudnell *et al.* 2002), the general hypothesis of our initial study (Shoemaker, 2003) was that SBS was a chronic, biotoxin-associated illness (CBAI, Shoemaker *et al.* 2001; Shoemaker *et al.* 2001) caused by exposure to water-damaged buildings (CBAI-WDB). The study design included screening of potential participants for confounding factors, a longitudinal, five time-point series of assessments, and the interventions of therapeutic treatment, removal from exposure, and re-exposure. Assessments included stan-

standardized recording of symptoms, measurements of visual contrast sensitivity (VCS) as an indicator of neurologic function, measurements of leptin and the hypothalamic hormone, alpha melanocyte stimulating hormone (MSH), as markers for illness, and cholestyramine therapy (CSM) to enhance toxin elimination rates. An effective therapeutic approach for enhancing toxin elimination rates allowed the demonstration of illness resolution with therapy, continued good health without re-exposure, relapse with re-exposure without prophylactic therapy, and recovery with re-treatment (Shoemaker *et al.* 2003). The results indicated that CBAI-WDB could be defined as a syndrome involving multiple-system symptoms, a neurologic functional deficit, and biochemical abnormalities.

VCS has been used for many years as a marker for neurotoxicity and has shown high sensitivity to effects caused by exposure to other biologically produced neurotoxins (biotoxins), including those produced by dinoflagellates (Shoemaker *et al.* 2001; Shoemaker *et al.* 2001), cyanobacteria (Shoemaker *et al.* 2000), spirochetes (Shoemaker *et al.* 2002), and apicomplexans (Shoemaker *et al.* 2002). VCS is a non-invasive, bedside measure of the contrast threshold at which the visual system can detect sinusoidal bar patterns of different sizes or spatial frequencies (i.e., cycles of dark and light bars per degree of visual arc). VCS deficits due to biotoxin exposure are greatest at 6-12 cycles per degree of visual arc, and resolved with CSM therapy (Shoemaker *et al.* 2001; Shoemaker *et al.* 2001; Shoemaker *et al.* 2000, Shoemaker *et al.* 2002; Shoemaker *et al.* 2002, Hudnell *et al.* 2002). CSM is a non-absorbable, anion-binding resin, used in doses FDA-approved for treatment of hypercholesterolemia, binds many biologically- and synthetically- produced toxins (Cohn *et al.* 1978; Mutter *et al.* 1988; Rateau *et al.* 1986; Brouillard *et al.* 1990; Creppy *et al.* 1995; Kerkadi *et al.* 1998; Underhill *et al.* 1995; Dahlem *et al.* 1989).

METHODS

We hypothesized that patients with chronic illness associated with exposure to water damaged buildings and the potential for formation of bioaerosols including, but not limited to mycotoxins, would present the same characteristics as those seen in patients with other CBAI. These characteristics include symptoms, VCS deficits, response to CSM therapy, lack of production of a protective immune response that would result in resolution of illness with removal from exposure, reacquisition of illness with re-exposure and presence of multiple biomarkers, including the genetic basis of susceptibility and markers for excessive release of pro-inflammatory cytokines.

156 consecutive symptomatic patients with exposure to 150 different indoor environments with water intrusion and musty smells, or visible mold growth or documented presence of toxigenic fungal species and without confounding biotoxin

exposures, coming to a specialty clinic 1/02-4/03 for treatment of chronic illness were enrolled in an open label treatment protocol. A control group of 111 patients, without exposure to WDB, was recruited as the first patient, matched for age and sex, coming to the clinic for a physical exam after a study patient was enrolled. Study patients served as their own controls for each of the 5-steps of the repetitive exposure trial and were compared to the clinic control group. Each patient was interviewed, with a complete medical history, symptoms recording, tobacco use, VCS testing, physical exam, pulmonary function testing (PFT), electrocardiogram and diagnostic lab studies, including HLA DR by PCR, MSH, leptin, MMP9, ACTH, cortisol, ADH, osmolality, testosterone, DHEA-S, androstenedione, MBP, tumor necrosis factor alpha (TNF), c-reactive protein (CRP), sedimentation rate (ESR), and aerobic culture of deep nasal space, were performed. Building exposures, VCS and symptoms review in control patients were recorded. All patients with an acute medical problem not related to exposure to water damaged buildings were excluded from the treatment and control groups. All patients with known occupational exposure to solvents, metal fumes and metal dust, hydrocarbons, ongoing neurologic disease, active Lyme disease, possible estuarine associated syndrome (PEAS), exposure to toxigenic cyanobacteria, and alcoholism with chronic liver disease were excluded. For those patients meeting the case definition of CBAI-WDB (Table 1), CSM was prescribed (Shoemaker *et al.* 2001; Shoemaker *et al.* 2001).

Table 1. Case definition of CBAI-WDB

SBS Case Definition

1. We propose that a definition of a case of SBS include each of the following elements:

 - potential for exposure to buildings with documented presence of toxigenic fungi, evident fungal growth or a history of water intrusion with musty smells;
 - presence of multiple symptoms in at least 4 of 8 system categories
 - absence of confounders

2. The case definition continues with at least 3 of following 6 criteria

 - VCS deficits
 - MSH deficiency
 - MMP9 elevation
 - HLA genotype

- ADH/osmolality dysregulation, measured simultaneously
- ACTH/cortisol dysregulation, measured simultaneously

3. The final criteria for case management include 2 of 3 of the following

- Response to CSM, with abatement of symptoms and resolution of VCS deficit to control levels
- Reduction of leptin, if elevated, with treatment
- Reduction in MMP9, if elevated, with treatment

4. Clinical note needs to be made of

- Presence of MARCoNS in deep nasal spaces
- Elevated levels of myelin basic protein antibodies

Patients were divided into three groups by location of exposure: 1) Consult (n=40); patients with potential for exposure to bioaerosols, who would not be returning for follow-up, 2) Residential (n=62); patients with potential for residential exposure to bioaerosols, with follow-up 3) Occupational (n=54); patients with potential for occupational exposure to bioaerosols, with follow-up. Baseline symptoms and VCS were compared for each group. Patients were then re-grouped for comparison by specificity of exposure, independent of residential/occupational exposure: 1) definitive fungal identification (fungal) (n=88), 2) presence of visible mold growth (growth) (n=48), 3) presence of water intrusion and musty smells (water) (n=20). Symptoms and VCS are compared for each group. Patients returning for follow-up care were evaluated with symptoms and VCS in sequential steps.

For patients with ongoing exposure to buildings with the potential for bioaerosols contamination, a 5-step exposure protocol was employed. Patients were evaluated at baseline (BASE), then treated until resolution of VCS deficits and symptoms were both obtained (AC-1). Patients were then kept away from the putative source of bioaerosols exposure, off CSM for 7 days (HOC). With informed consent, patients were returned to the suspect building, with re-evaluation in 3-5 days (BOC). Following documentation of symptoms and VCS, patients were then re-treated with CSM (AC-2), with final recording of all parameters. A subset of exposed patients elected to continue exposure in the suspected building, with ongoing use of CSM in prophylactic doses of one scoop of CSM taken twice a day (Prophyl). Vision tests and statistical analyses were performed as previously (Shoemaker *et al.* 2001; Shoemaker *et al.* 2001). LabCorp and Esoterix performed laboratory testing, both CLIA approved, high complexity, national laboratories.

RESULTS

VCS scores and symptoms are each similar in all 6 groups at baseline and each is markedly different from non-exposed, control patients, $p < .001$ (Figure 1-5). Treatment results in resolution of VCS deficits and reduction of symptoms to levels of controls. There is no difference in biomarkers at baseline between the groups. Exposure to defined fungal species showed no difference in symptoms or VCS compared to exposures to visible mold growth, water intrusion and musty smells. Reduction of leptin with treatment was seen in 85%, and MMP9 reduction was seen in 90% of patients.

Figure 1. Controls vs. initial illness: Occupational exposure, residential exposure, consultation but no treatment

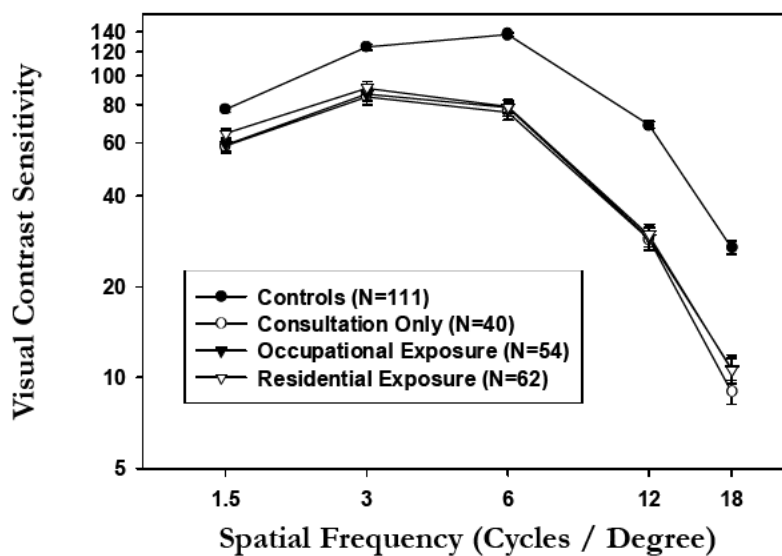


Figure 2. Controls vs. initial illness: Fungi genera identified, visible evidence of fungi only, water damage evidence only

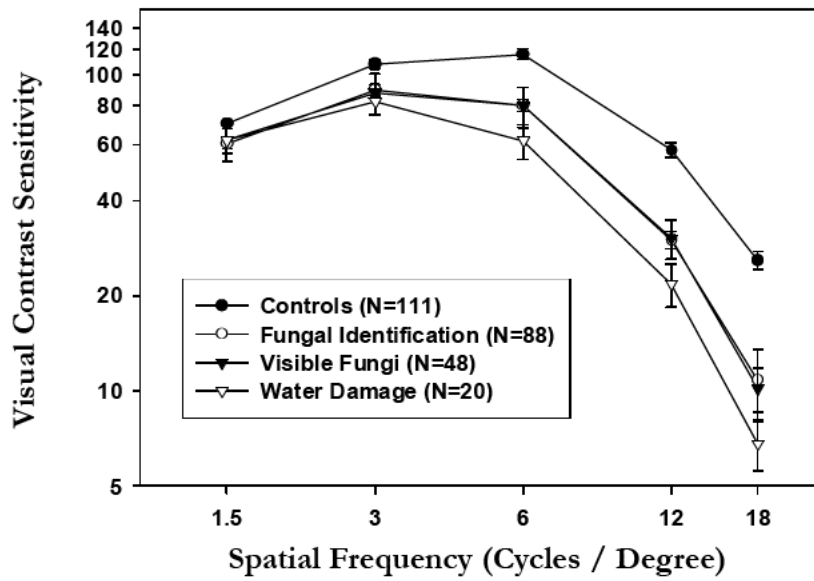


Figure 3. Occupational cohort: time series

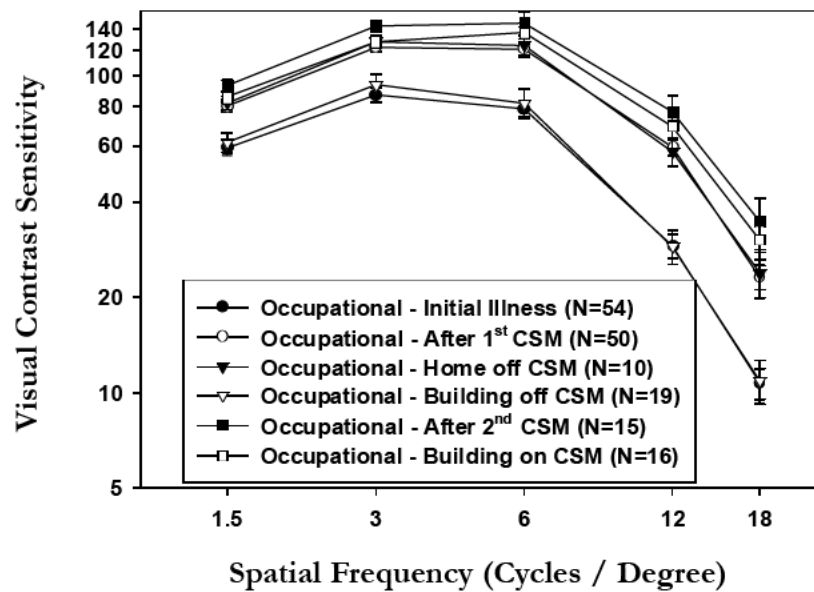


Figure 4. Residential cohort: time series

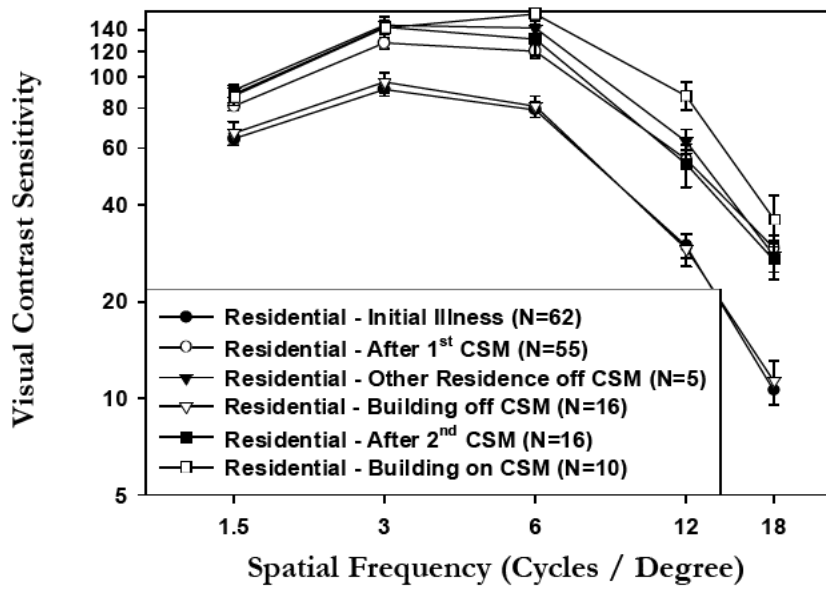
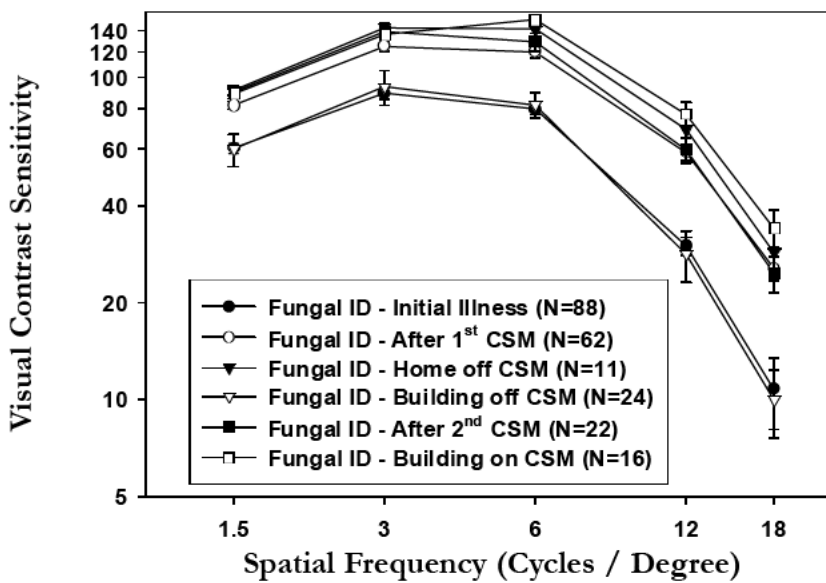


Figure 5. Fungal identification cohort: time series



Marked differences in symptoms and VCS scores were seen with the 5-step protocol. Base scores approximated BOC scores (figures 1, 2, 3, 4, 5). AC-1 and AC-2, as well as Prophyl were essentially identical and equal to controls in both symptoms and VCS. HOC approximated the AC-1 group. Figures 1, 2, 3 show no differences between Base for consult, residential or occupational groups. Figures 2, 3 show no differences between residential or occupational groups for each of the six time steps. Figure 4 shows no differences between before and after treatment for groups of patients exposed to defined fungal genera, visible mold growth or those exposed to water intrusion and musty smells.

HLA DR by PCR showed a strong relative risk (>2.0) for illness for a select group of genotypes (Table 2), specific, non-specific or protective. The rapid re-acquisition of illness, with VCS changes noted, in patients with high relative risk genotypes within 3-5 days of re-exposure to the same building, with no changes in water intrusion in the building, strongly argues against a linear, dose-response relationship as the operative factor in illness acquisition. HLA susceptibility was present in over 75% of cases.

MSH deficiency was present in over 92% of cases in all groups, correlating closely with symptoms and VCS deficits. Leptin elevation was found in 60% of affected patients; a better measure of the importance of leptin as a marker was change in leptin levels with treatment. ADH/osmolality, ACTH/cortisol and androgens showed marked dysregulation before treatment, markedly different from controls. MMP9 showed significant elevations at baseline, with return to normal in over 90% of affected patients following treatment.

Table 2. Susceptible genotypes

		RR
Fungal Only	7-2-53	4.6
	13-6-52A,B,C	3.4
	17-2-52A	3.5
Multiple	4-3-53	2.1
	11/12-3-52B	4.6
	14-5-52B	2.0
Post-Lyme	15-6-51	2.1
Dinoflagellate	4-8-53	2.6

VCS showed dynamic changes, in-step with treatment and re-exposure in other biotoxin illnesses (Cohn *et al.* 1978; Mutter *et al.* 1988). VCS had a low false negative rate (7%) and no false positives. VCS showed correction in 95% of treated patients. The role of second hand smoke as a confounder in diagnosis from water-damaged buildings is not supported by this study.

DISCUSSION

These data support the concept that the illness in patients exposed to bioaerosols in water-damaged buildings is readily identified as a CBAI. CBAI-WDB is a complex clinical condition that demonstrates inflammatory, hormonal and neurotoxicological biomarkers found in other chronic, biotoxin-associated illnesses. The treating physician can recognize the illness at the bedside, rule out confounding diagnoses, perform VCS and document the multiple biochemical abnormalities present in patients to satisfy the three tiered case definition (Table 2). The large treatment group in this study documents the utility of CSM as a therapeutic agent, as well as for prophylactic use. CBAI-WDB does not follow a dose-response relationship, but is associated with genetic susceptibility factors in HLA DR (Shoemaker *et al.* 2003; Shoemaker *et al.* 2002).

CONCLUSIONS

This study demonstrates the benefits of approaching chronic illness acquired by individuals with exposure to water damaged buildings as a CBAI. A registry of biomarkers, documentation of therapeutic benefit from CSM, a benign, FDA approved medication, and documentation of prevention of relapse in susceptible, exposed patients using CSM is consistent with other CBAI. VCS is a useful, inexpensive, non-invasive, portable, reproducibly reliable, bedside diagnostic test of significant utility in all 5 steps of the repetitive exposure times. MSH, leptin, MMP9, MBP, pituitary hormones, taken together with symptoms, HLA, and VCS, all contribute to the diagnostic basis of CBAI-WDB and provide the basis for follow-up of affected patients. The benefits of VCS and symptoms recording in screening large populations and building-wide prevalence studies will await completion of further studies, currently in progress. A double blinded, placebo controlled clinical trial that will add confirmation to these results is underway.

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CLINICAL EVALUATION OF SUSPECTED MOLD NEUROTOXICITY

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ABSTRACT

Doctors and forensic scientists are increasingly being challenged to evaluate individuals with mold exposure for health injuries, such as neurotoxicity. A significant body of research, described below, shows possible mold neurotoxic effects. Individuals presenting with neurotoxicity can be effectively evaluated with a standard neuropsychological evaluation, as follows. A 47-year-old married nurse, exposed to numerous molds over a 10-year period, with periods of peak exposures, became ill with multi-system symptoms, referable to respiratory, autonomic and central nervous system function. Carpeting dust analysis over various locations in the office showed elevated mold levels, including *Stachybotrys*.

Work and home history did not indicate mold or other neurotoxic substance exposure, other than mold from office. Immune function testing found elevated antibodies to *Stachybotrys* and other molds. Neuropsychological testing found deficits in working memory, processing speed, word fluency, manual dexterity, visual perception and executive function. Emotional function and personality were generally within normal limits. Although evaluated by many medical specialties, no competing diagnosis was found, resulting in a probable diagnosis of mold neurotoxicity.

INDEX TERMS: Adverse effects, mycotoxins, neurotoxicity, neuropsychology, toxic mold

INTRODUCTION

Objective: To show diagnostic procedures for analyzing an individual case with suspected mold neurotoxicity affecting central nervous system function.

Mycotoxins are formed by the hyphae and spores of common molds growing under a variety of conditions. Other prominent compounds include volatile organ-

ic compounds (VOC's), alcohols and aldehydes – these are not mycotoxins, although with sufficient concentration, they could have neurotoxic effects. Mycotoxins and possibly other by-products may be responsible for symptoms of headache, dizziness, and eye and mucous membrane irritation among individuals in fungus-contaminated buildings (Levetin, 1995).

Mycotoxin exposure at doses capable of producing chronic disease are usually far below those responsible for acute effects (Samson, 1992). Some are “neurotoxins, which in low doses may cause sustained trembling in animals, but at only slightly higher doses cause permanent brain damage or death” (Samson, 1992).

Case reports and other medical evidence of mold neurotoxicity: Johanning and Landsbergis (2001) reported that a majority of patients with fungal exposure had at least three nervous system complaints (such as headaches, nervousness, concentration problems, dizziness, and excessive fatigue) with 77 percent reporting at least one CNS symptom. Chronic fatigue was found in 50% of the subjects. Craner (2001) reported neuropsychological symptoms following mold exposure, including a case where the subject developed “marked behavioral changes, difficulty concentrating and short-term memory impairment, and profound fatigue,” with neurocognitive impairment persisting after removal from the contaminated house. Auger (2001) reported four cases of chronic toxic encephalopathies apparently related to exposure to toxigenic fungi. Sigsgaard (2001) reported neurological and neuropsychological symptoms increasing with the number of hours spent in a damp building. The symptoms included headache, tiredness, and sleeping difficulties. Gordon (2001) studied twenty people who reported cognitive changes following exposure to fungi. All subjects were found to be neuropsychologically impaired on at least one measure, with 65 percent of the sample meeting three or more of the impairment criteria.

In summary, there is significant support in the scientific literature (including descriptions of mechanisms, group studies, and individual neuropsychological evaluations) for diagnosing individual cases of fungal/mold neurotoxicity. In the past, neuropsychological analysis has been helpful for the diagnosis of individual cases with numerous types of neurotoxic substance exposures (Singer, 1990; Singer, 2003a; Singer, 2003b).

METHODS

A standard neurobehavioral toxicology protocol was utilized, including extended history-taking, record review, and neuropsychological testing.

Subject: A 47 year old white woman, with 16+ years of school and a B. S. in Nursing, married with 5 children, was referred by her doctor for further evaluation

following mold exposure and subsequent illness. She was unemployed and in a worker's compensation case. Her main symptoms were deteriorated memory, concentration and learning skills; difficulty with multi-tasking; fatigue; and need for excessive sleep (10 hours per night). She was examined 8 months after workplace exposure ceased.

Exposure and symptoms: The subject worked a 40-hour week from June 1991 through February 2001 as a visiting nurse, spending 3-5 hours per day in the same office. She brought materials and clothing from the office to her car to visit clients on a daily basis. In 1994, after office renovations, there were plumbing and roofing leaks, resulting in sagging ceiling tiles. Buckets were placed to catch further precipitation from the ceiling, with the area blocked off with chairs. Although the exterior roof underwent various repairs, the roof continued to leak, and there was no remediation of the water from the roof, affecting the interior ceiling, walls, or carpet. The roofing leaks began to spread in the office with repeated water intrusions.

In 1995, the subject began experiencing bladder incontinence, fatigue, numbness and tingling of her heels and face, leg twitching at night, and dizziness, which occasionally progressed to true vertigo. She then developed bowel incontinence. She was examined by numerous medical specialists, who found no medical cause for her condition. She began to have significant fatigue all the time, with frequent bouts of colds and influenza. The symptoms continued through 1999, at which time the room in her office that had been blocked off because of leaks was reopened and reconnected with the general offices. All of the office and patient supplies were placed in this room, and conferences, staff meetings, and in-service meetings were held there, so there was significant potential exposure to this patient when she was in this room. In 2000, her desk was placed next to the open doorway of this room. She quickly and progressively became more ill, with confusion, and was in three mild automobile accidents (with no head trauma) within three months. She developed a chronic sinus condition, with a bronchial cough. She felt better on the weekends. Other coworkers also were reportedly sick, at least one with blood indicators of mold exposure, such as those of *Stachybotrys* exposure.

In January 2001, elevated levels of molds were found in the office (see below). The staff moved their own office supplies to another office, which had been vacated and remodeled because of toxic mold problems. In February, her condition worsened, with significant difficulties in automobile driving and concentrating, so she asked for medical leave.

RESULTS

Environmental testing: On 1/5/2001, fungal air and surface testing of the facility was conducted. The employees in the problem building surveyed their symp-

toms, refused to work in the contaminated building, and presented the results, which were included in the environmental testing report. The most common complaints among co-workers, all nurses or other health care workers, were respiratory difficulties, headaches, sleepiness, decreased concentration, sluggishness, irritability, anxiety, and mental sluggishness. Other reported symptoms included muscle twitching and jerking, clumsiness and incoordination, extreme sensitivity to odors and light, nosebleeds, and burning itchy eyes and skin.

The left half of the floor in the women’s rest room was 80-100 percent saturated with water; the other half of the floor was at 20-40 percent relative saturation. The ceiling tiles were stained in the locations where roof leaks were reported. The environmental survey revealed that substantial quantities of *Stachybotrys* and other mold spores and contaminated dust were released during the demolition/construction work.

Carpet dust composite analysis: This method is expected to reflect a relatively long-term “record” of particles that have been airborne in the offices, and is thought to be the best single record of the levels of exposure throughout the office over the time that the subject was present. The sample was collected as a composite on one occasion, with some of the dust drawn from the carpet in several locations around the office which would normally be difficult to reach with a vacuum cleaner and might therefore be long-term repositories of dust

Table 1. Carpet dust composite analysis

Fungus type	Non-viable counts per 100 mg of carpet dust
<i>Stachybotrys</i>	200
<i>Aspergillus/ Penicillium</i>	400
<i>Cladosporium</i>	600
<i>Basidiospores - phaeo</i>	700
Summary total	3200

Medical testing results: Brain MRI and NCV were reported as normal. Based on reports of numerous medical specialists, no medical cause of her illness has been confirmed, other than the fungal exposure. Significant fungal antibodies were found in the subject’s blood, as follows (all units are reported as ELISA by Immunosciences Lab, Inc.):

Table 2. Fungal and myelin antibody testing

Date	Type	Result	Norms
11/28/00	IgG <i>Stachybotrys</i>	3100	0-1600
11/28/00	IgG <i>Cladosporium herbarum</i>	5500	0-1600
11/28/00	IgG <i>Pullularia pullulans</i>	4900	0-1600
4/10/01	IgM Myelin basic protein	55	0-50
7/17/01	IgG <i>Penicillium notatum</i>	4500	0-1600
7/17/01	IgG <i>Pullularia pullulans</i>	3300	0-1600
7/17/01	IgG <i>Stachybotrys</i>	2100	0-1600

Neuropsychological testing results

Symptom Testing: Positive on the Neurotoxicity Screening Survey (Singer, 1990), which assesses the consistency of responses to that of subjects with diagnosed neurotoxicity.

Cognitive and Executive Function Testing: The subject was tested on two occasions by different neuropsychologists (using different tests), as she sought further consultation from a neurotoxicologist. The significant findings are as follows:

Pre-exposure IQ ¹	95 th percentile
WAIS-III Working Memory	25 th percentile
Processing Speed	32 nd percentile
WMS-III Auditory	47 th percentile
WMS-III Auditory Delayed	42 nd percentile
WMS-III Visual Delayed	58 th percentile
WMS-III General Memory	58 th percentile
Controlled Oral Word Association Test	10 th percentile
Grooved Pegboard Test	8 th and 9 th percentiles
Paced Auditory Serial Addition Test	<1 st percentile
Benton Visual Retention Test	Indicates Acquired Impairment
Stroop Color and Word Test	4 th percentile (Color/Word)
Army Trail Making Test, part B	21 st percentile
Visual Search and Attention Test	1 st percentile

Emotional Testing: Emotion was within normal limits, as measured on six scales of emotional function.

Distortion and Malingering Testing: Based on nine negative indicators, as well as her social history, malingering is unlikely to be a significant factor.

Personality Testing: Using the Neo Personality Inventory Revised, and comparing the results with her social history, the subject was found to probably have a deterioration of personality, although definite abnormality was not reached.

DISCUSSION AND CONCLUSION

With regard to time- or concentration-dependent exposure indicators of mold exposure, scientists cannot intentionally expose people to mold, mycotoxins and other products of damp buildings, while constantly monitoring levels of exposure, in order to have an exact determination of exposure, because that would be unethical. Therefore, the carpet samples and biomarkers provide some of the best possible indicators of exposure.

The excessive antibody production to multiple toxic molds found in the patient's blood probably means excessive exposure, especially in light of the patient's severe symptoms. In addition, the composite carpet sample provided a good indicator of long-term exposure. The carpet sample would reflect the chronic exposure; however, there were periods of acute exposure resulting from building renovations releasing mold spores and mold parts, of which we have no specific environmental measurements, as is probably always the case; measurements are almost never considered until after people have become seriously ill.

The immune function testing found elevated antibodies to many of the same excessive levels of molds identified in the carpet sample. These molds were capable of producing the symptoms found, based upon the literature cited in this report.

The findings are most consistent with mold neurotoxicity. The subject reported no occupational or household exposure to pesticides, neurotoxic substances, no head injuries or other serious injuries, or diagnoses of psychiatric disorders. It is unlikely that the results reflect senile dementia, as her vocabulary and reasoning skills (which deteriorate with dementia) were at the 95th percentile, and the onset of symptoms was at age 39. Depression was ruled out by the Beck Depression Inventory. No competing diagnoses were found in the extended medical record. Brain MRI was normal, ruling out stroke, tumor, etc. Other workers were reported to be ill with symptoms resembling mold toxicity, by self report. The proposed method has provided substantial evidence, within reasonable scientific certainty, that the subject's central nervous system has deteriorated following extended exposure to various molds.

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THE CHRONICITY OF COGNITIVE IMPAIRMENT ASSOCIATED WITH EXPOSURE TO TOXIC MOLD

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INTRODUCTION

Gordon *et al.* (1999) reported an association between exposure to toxic molds and cognitive impairment. Since then, two other studies (Baldo, Ahmad, Ruff, 2002; Gordon, *et al.* 2004) have offered further support for such an association. While some efforts have been made to examine the chronicity of cognitive symptoms in samples of persons exposed to mold (e.g., Sudakin, 1998), there are no studies in the literature to date which examine patterns of performance over time on comprehensive batteries of neuropsychological tests. Examining chronicity is crucial to determining whether the cognitive impairments found to be associated with mold exposure persist for significant periods of time after exposure has ceased. The present study was conducted in order to begin to address this issue.

METHOD

A group of eight individuals with histories of exposure to toxic mold, who were originally administered a battery of neuropsychological tests by the senior author, were re-tested at an interval of one to five years after the initial testing. All eight were exposed to toxic molds such as *Stachybotrys atra*, *Penicillium*, and *Aspergillus* for varying lengths of time. All exposures were documented by environmental testing conducted during the exposure period. All exposures had ceased by the time of the first testing.

Seven of the eight persons tested were women. At the second testing, they ranged in age from 37 to 65 ($M = 49.25$, $SD = 10.12$). Most were college graduates (Mean years of education = 15.75, $SD = 1.95$). Participants' full scale IQs varied from the average to very superior ranges ($M = 116$, $SD = 11$). All eight continued to report symptoms of cognitive impairment at re-testing.

RESULTS

Because group data do not capture the full extent of the chronicity of cognitive impairments in a sample of this size, especially in higher functioning individuals, a brief description of each participant's functioning is provided below. In each case, although impairments were chronic in some domains, there was some fluctuation in test scores between evaluations. Some scores improved, some remained unimpaired, and some worsened. Because a thorough discussion of test and retest scores for each subject is beyond the scope of this paper, we have chosen to focus on domains where chronic impairments (either relative or absolute) were found at re-testing. Key supporting data for chronicity are provided in Table 1:

Participant 1 (Evaluated 1998 and 2002): Participant 1 is a professional with a master's degree. She continued to experience reduced processing speed, memory, and learning difficulties.

Participant 2 (Evaluated 1997 and 2001): Participant 2 graduated from college *cum laude*, with honors in two majors. Until the onset of her cognitive impairments, she worked in a literary field. At re-testing, verbal learning and memory continued to be reduced and verbal memory and IQ remained inconsistent with her levels of academic performance in college. Some difficulties with executive functions also remained at the second testing.

Participant 3 (Evaluated 2001 and 2002): Participant 3 continued to have significant impairments in the domains of attention and concentration and verbal learning at re-evaluation.

Participant 4 (Evaluated 2000 and 2002): On re-testing, Participant 4's test results continued to indicate reduced processing speed on cognitively demanding tasks.

Participant 5 (Evaluated 2001 and 2002): Participant 5's IQ is in the very superior range. Re-testing indicated the continuation of significant impairment on tests of memory function relative to IQ level. Difficulties on tests of executive functions also remained.

Participant 6 (Evaluated 2000 and 2002): At the second testing Participant 6 continued to have significant difficulties on tests of complex visual memory and impaired executive functions.

Participant 7 (Evaluated 1997 and 2002): Participant 7 is a college graduate who has a successful work history in the teaching, management, and financial fields. Re-test findings showed continued decreased intellectual functioning, impaired visual

memory on complex tasks and reduced performance in aspects of executive functioning.

Participant 8 (Evaluated 1997 and 2002): Participant 8's IQ is in the superior range. On re-testing, relative impairments on tests of attention and concentration, visual memory, and verbal encoding were noted to have continued.

In all eight cases, a thorough review of relevant medical history revealed no preexisting problems that could account for the findings of cognitive impairment (e.g., traumatic brain injury, neurological disorders, significant psychiatric history) or for their chronicity.

DISCUSSION

These data indicate that, despite some variations in test findings, all participants continued to experience cognitive impairment one to five years after initial neuropsychological testing and termination of mold exposure. The findings suggest that some cognitive deficits secondary to toxic mold exposure are chronic in this small sample of individuals, even after cessation of mold exposure.

Table 1. Neuropsychological Testing Data^a from Study Participants Documenting Chronicity of Impairment from Initial Testing to Subsequent Re-testing

Participant	Percentile Scores on Testing 1	Percentile Scores on Testing 2
1	<p>Processing Speed WAIS-III^b Processing Speed = 21%ile</p> <p>Memory & Learning WAIS-III Working Memory = 9%ile WMS-III^c Auditory Immediate = 13%ile WMS-III Auditory Delayed = 23%ile WMS-III Auditory Recognition = 25%ile WMS-III Visual Immediate = 7%ile WMS-III Visual Delayed = 50%ile WMS-III Immediate memory = 5%ile WMS-III General memory = 27%ile WMS-III Working memory = 5%ile CVLT^d Trials 1 to 5 = <1%ile</p>	<p>Processing Speed WAIS-III Processing Speed = 21%ile Stroop^e Word = 3%ile Stroop Color = 1%ile Trails A^f = 6%ile</p> <p>Memory & Learning WAIS-III Working Memory = 18%ile WMS-III Auditory Immediate = 9%ile WMS-III Auditory Delayed = 23%ile WMS-III Auditory Recognition = 37%ile WMS-III Visual Immediate = 3%ile WMS-III Visual Delayed = 7%ile WMS-III Immediate memory = 3%ile WMS-III General memory = 13%ile WMS-III Working memory = 8%ile CVLT Trials 1 to 5 = 2%ile</p>
2	<p>Intellectual Functioning WAIS-III Verbal IQ = 82%ile</p> <p>Verbal Learning & Memory WMS Verbal Memory Index = 97 (vs. Visual Memory Index = 135) CVLT Trial 5 = 2%ile CVLT Trials 1 to 5 = 24%ile All other CVLT scores = 16%ile</p> <p>Executive Functions Booklet category test = Could not complete</p>	<p>Intellectual Functioning WAIS-III Verbal IQ = 77%ile WAIS-III VIQ < WAIS-III PIQ (p < .05)</p> <p>Verbal Learning & Memory Auditory Immediate Memory = 63%ile</p> <p>All CVLT scores = 50%ile</p> <p>Executive Functions Booklet category test = 8%ile</p>

	Percentile Scores on Testing 1	Percentile Scores on Testing 2
3	<p>Attention & Concentration CPT[®] Overall Index = 10.67 (Borderline) CPT Confidence Index for Attentional Problems = 93% Verbal Learning CVLT Trial 5 = 2% CVLT Long Delay Free Recall = 2%</p> <p>Processing Speed All PASAT[™] Scores < 1% WAIS-III Processing Speed Index = 21% Purdue pegboard scores = < 2%</p>	<p>Attention & Concentration CPT Overall Index = 10.67 (Borderline) CPT Confidence Index for Attentional Problems = 93% Verbal Learning CVLT Trial 5 = 6% CVLT Long Delay Free Recall = 6%</p> <p>Processing Speed All PASAT Scores < 2%</p>
4	<p>Memory All WMS-III indexes except Working memory < WAIS-III VIQ (p < .05). All CVLT scores = 16 or 18%</p> <p>Executive Functions Booklet category Test = 42% Stroop Color/Word = 65%</p>	<p>Memory WMS-III Verbal Immediate and Delayed Memory < WAIS-III VIQ (p < .05). WMS-III Visual Immediate and Delayed Memory < WAIS-III PIQ (p < .05).</p> <p>Executive Functions Booklet category Test = 1% Trails B = 50% Stroop Color/Word = 58%</p>
5	<p>Visual Memory WMS-III Visual Immediate and Visual Delayed < than FSIQ (p < .05) CVLT Trials 1 to 5 < 16%</p> <p>Executive Functions Watson Glaser = 25% Booklet category test = 4%</p>	<p>Visual Memory All Rey Complex Figure scores = < 1%</p> <p>Executive Functions Watson Glaser = 5% IOWA[®] reading comprehension = 30% (extra time required)</p>

	Percentile Scores on Testing 1	Percentile Scores on Testing 2
7	<p>Intellectual Functioning WAIS-R PIQ = 27^oile</p> <p>Visual Memory WMS-R^m Visual Reproduction = 10^oile</p> <p>Executive Functions</p> <p>Watson Glaser = 3^oile Booklet category test = Could not complete</p>	<p>Intellectual Functioning WAIS-III PIQ = 34^oile</p> <p>Visual Memory Rey Complex Figure = <1^oile</p> <p>Executive Functions IOWA reading comprehension = 16^oile IOWA reading efficiency = 6^oile Watson Glaser = 10^oile Stroop Color Word = 24^oile</p>
8	<p>Attention & Concentration WMS-R Attention/ Concentration Index Score = 89</p> <p>Visual Memory WMS-R Visual Memory Index = 113</p> <p>Verbal Encoding CVLT Trial 1 = 50^oile</p>	<p>Attention & Concentration CPT Clinical Significant Attention Problem, Confidence Index = 99.9%</p> <p>Visual Memory WMS-III Visual Immediate Memory < WMS-III Auditory and Visual Delayed Memory (p < .05)</p> <p>Verbal Encoding CVLT Trial 1 = 50^oile</p>

*Only data indicative of significant impairments (absolute or relative) where performance in the given domain did not change significantly from Testing 1 to Testing 2 are included, ^b Wechsler Adult Intelligence Scale - Third Edition, ^c Wechsler Memory Scale - Third Edition, ^d California Verbal Learning Test, ^e Stroop Color Word Test, ^f Trail Making Test - Part A, ^g Conners Continuous Performance Test, ^h Paced Auditory Serial Attention Task, ⁱ Trail Making Test - Part B, ^j Watson Glaser Critical Thinking Appraisal - Form B, ^k Iowa Silent Reading Test, ^l Wechsler Adult Intelligence Scale - Revised, ^m Wechsler Memory Scale - Revised

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Chapter 3

Health Effects III – Health Assessment & Case Studies

Session chairs:

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HEALTH EFFECTS OF MOISTURE DAMAGE ASSOCIATED MICROBES

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ABSTRACT

Moisture damage and microbial exposure are one of today's most common causes of poor indoor air quality and health complaints in residential and office buildings, day care centers, schools and hospitals. 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, mites together with excess temperatures and insufficient ventilation. In a problem building the harmful exposure is a mixture of dusts, vapors and finally microbial growth on moist surfaces and damp construction materials. These microbes are molds and yeasts and various bacteria, e.g. actinomycetes and atypical mycobacteria. These microbes produce spores and various kinds of mold toxins, bacterial endotoxins and microbial volatile organic compounds (mVOC) that may all have adverse effects to human health. The objective of this review is to conclude the current knowledge on the various health effects of indoor air microbes as shown in epidemiological research and shortly review also the pathophysiological processes behind these health effects.

KEYWORDS: molds, asthma, allergy, alveolitis, infectious disease

EXPOSURE

Temperature, relative humidity, draught and odors affect the perception of indoor air quality and modify the effects of chemical and microbiological contaminants. In buildings with moisture damage and microbial growth, the exposure is always a complex mixture of particles and volatile chemicals. In practical situations, it is impossible to measure all possible components and to distinguish between the effects of various exposures. Therefore, the exposure is often estimated by limited number of surrogates such as microbial counts in material samples or VOC-meas-

urements in the air. In practical situations of risk assessment, it is necessary to quantify both the exposure and the effect, and still a certain amount of uncertainty will remain when causal relationship and the health risk are estimated.

The human body reacts to a number of exposing agents in a nonspecific way, and the reactions are often similar no matter if the exposure in question is a chemical, inorganic dust or biological agent, such as microbes. In addition, knowledge on additive or synergistic effects and cross-reactivity between microbes is vague.

Fungi produce spores in certain phase of their life cycle. In addition to spores, also mycelia and organic compounds released by the microbial colony are harmful to health. Molds and yeast build colonies in any surroundings where moisture is present continuously or repeatedly. Actinomycetes, e.g. *Streptomyces* and *Mycobacteria* are soil bacteria that grow in environmental conditions similar to those of fungi. Most of the studies published recently concern the health effects of molds and actinomycetes, but there is growing interest towards other microbes such as *Mycobacteria*, microbial toxins and mites.

The most common outdoor molds are *Cladosporium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Candida* and *Botrytis*. The most prevalent indoor molds in non-damaged houses are *Penicillium*, *Aspergillus* and *Cladosporium* (Ledford, 1994). In Nordic countries, *Cladosporium* is the most common mold in outdoor air while the concentrations of *Alternaria* are much lower than in Middle Europe. These spores can be found indoors on surfaces and in settled dust but they start growing only when sufficient moisture is available.

When a building is damaged with moisture or water leaks the common microbes are replaced by molds and other microbes requiring higher water activity in the growth media. The occurrence of these microbes on surfaces or in construction indicates damage and health risk in the building. The airborne concentrations of these mold spores are not necessarily high in water damaged buildings. Sometimes low concentrations of unusual species may give a hint of hidden mold growth in the building. Airborne mold concentrations and health effects do not correlate well and therefore surface and material samples are preferred as measures of exposure instead of air samples (Dillon *et al.* 1996; Davies, 1995; Husman, 1999). A list of indicator microbes typical of water damaged buildings has been given in 1992 by an expert group (Samson *et al.* 1994). It is obvious that these kinds of lists are inconclusive.

HEALTH EFFECTS OF INDOOR AIR MICROBES

The health effects of indoor air microbes, molds and actinomycetes can be divided into five categories: 1) irritation symptoms, 2) respiratory infections, 3) allergic

diseases, 4) pulmonary diseases such as alveolitis, organic dust toxic syndrome (ODTS) and chronic bronchitis. These categories are artificial and partly overlapping. The definitions of diseases and symptoms vary in different studies and different countries and languages (e.g. alveolitis vs. hypersensitivity pneumonitis).

Irritation and Nonspecific Symptoms: Several irritation symptoms have been described in water damaged and damp buildings. The most prevalent symptoms are irritation of respiratory tract, such as rhinitis, cough, hoarseness, chest tightness and irritation of eyes (Brunekreef *et al.* 1989; Dales *et al.* 1991). In the Netherlands, mold exposure was associated mainly with lower respiratory symptoms, cough and phlegm production, and wheeze (Brunekreef, 1992). Similar findings were previously published by other groups in smaller study populations (Waegemaekers *et al.* 1989; Holmberg, 1984).

Also among children, the risk of prolonged rhinitis and cough was strongly associated with exposure to water damage in residential buildings and day care centers (Andrae *et al.* 1988; Koskinen *et al.* 1999). Among Finnish pre-school children, nasal congestion and excretion, persistent cough, upcoming phlegm and wheezing were significantly associated with dampness and molds in homes (Jaakkola, J. J. K. *et al.* 1993). In Finnish residential buildings, rhinitis, sore throat, hoarseness, prolonged cough and upcoming phlegm were significantly more prevalent among residents exposed to dampness and/or mold than among controls (Husman *et al.* 1993). The concentrations of airborne mold spores are generally not particularly high in water-damaged buildings, but they were higher in damp apartments than in control apartments and the fungal genera were different from those in control apartments (Hyvärinen *et al.* 1993).

Nonspecific symptoms like fatigue and nausea have also been associated with damp housing. Similar and in some cases even more severe and prominent nonspecific symptoms (e.g. chronic fatigue syndrome) have been described in a number of studies (Auger *et al.* 1990). In Britain, Platt and coworkers have described symptoms such as nausea, vomiting, backache and joint pain, together with respiratory symptoms associated with mold exposure (Platt *et al.* 1989). Headache, dizziness and difficulties in concentration have also been associated with mold exposure in a Finnish study (Husman *et al.* 1993). Non-specific symptoms such as fever and chills, headache, muscle and joint ache, dry cough and chest tightness belong also to symptoms of alveolitis (Pepys, 1969).

Respiratory Infections: The occurrence of common respiratory infections is higher in exposure to building mold. This finding is consistent both in adults and in children (Brunekreef *et al.* 1989; Waegemaekers *et al.* 1989). The etiologic agent of the infection can be any of the common respiratory pathogens, viruses causing

common cold and flu and secondary bacterial complications, such as sinusitis or acute bronchitis.

Among children, the attack rate of respiratory of infections was significantly higher among the exposed group of children (Husman *et al.* 1993). Similar results were found among children attending a moldy day care center (Koskinen *et al.* 1995). In addition to common colds, the occurrence of paranasal sinusitis and acute bronchitis was increased in the exposed group (Husman *et al.* 1993) The occurrence of infections in lower respiratory tract e.g. acute bronchitis have been higher in mold exposure situations (Pirhonen *et al.* 1996). The association between acute infection of the middle ear in children and mold exposure has also been shown in previous studies (Koskinen *et al.* 1999; Koskinen *et al.* 1995).

Actinomycetes and certain fungi, e.g. *Aspergillus* cause severe infections in human body. Aspergillosis has been described in bronchi, paranasal sinuses, vertebrae and in postoperational infections (Castelli *et al.* 1990; Bush, 1994;. Hantsch, Tanus , 1991; Schwartz *et al.* 1992; Shah *et al.* 1993; Bhagat *et al.* 1993; Sauter *et al.* 1994). Allergic bronchopulmonary aspergillosis (ABPA) can also be followed by invasive aspergillosis (Ganassini, Cazzadori, 1995). Invasive infections caused by molds are very rare in otherwise healthy persons but in most cases fungi cause opportunistic infections in organ transplantation patients, immunosuppressed patients and HIV-patients (Cendan *et al.* 1993; Seaton, 1993; Björkholm *et al.* 1994). Actinomycosis has been found in lungs, paranasal sinuses, in contaminated wounds contamination, and in intrauterine infections in IUD-users (IUD=intrauterine device) (Cendan *et al.* 1993; Hsieh *et al.* 1993). Skin lesions and dermatitis can also be caused by microbes typical to water damage in buildings (Bergman, Kauffman , 1984; Simpson, Nightingale, 1994). In Sweden, an increase in occurrence of invasive mycoses has been reported (Alestig *et al.* 1994).

The underlying mechanisms behind the association between mold exposure and increased occurrence of common respiratory infections are still largely unknown, but altered mucociliary function may be one possible explanation (Joki *et al.* 1993). Additionally, certain molds have immunosuppressive effects to animals in experimental exposure (Flannigan *et al.* 1991). Alteration of the immune defense system by microbial toxins may promote manifest infections by other infective agents. The excess of sinusitis episodes associated with mold exposure may be due to either mold allergy or other inflammatory and vascular processes causing nasal congestion (Wickern, 1993; Bush, 1994; Tsimikas *et al.* 1994;. Allphin *et al.* 1991; Loury *et al.* 1993;).

Allergic Diseases: Like most organic dusts, several molds are known allergens causing type-I allergic rhinitis, asthma and allergic conjunctivitis. *Alternaria*, *Cladosporium*, *Aspergillus*, and *Botrytis* commonly occurring in outdoor air are aller-

genic (Ledford, 1994; Peat *et al.* 1993; Gravesen, 1979). 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. Health effects of wood deteriorating fungi are less well known, but at least *Serpula sp.* (formerly called *Merulius*) is allergenic. It is impossible to give any complete list of allergenic microbes because new species are continuously detected and reported (Flannigan *et al.* 1991; Arundel *et al.* 1986; Salvaggio, Aukrust, 1981).

Fungal allergens are primarily found in the spores but also occur in other structures, e.g. mycelia. All identified fungal allergens are water-soluble glycoproteins, some of which are enzymes (Lopez *et al.* 1989). Only a few fungal allergens have been precisely characterized (Savolainen *et al.* 1990; Aukrust, Broch, 1979; Horner *et al.* 1988). 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.) (Nordvall *et al.* 1990; Potter *et al.* 1991).

The specificity, sensitivity and repeatability of skin tests are poorly documented. Both false negative and false positive test results are possible (Ledford, 1994; Savolainen *et al.* 1989). Only a limited number of mold extracts is currently available for diagnostic purposes. The cross-reactivity between different fungal extracts is not known, but some suggestions exist that cross-reactivity may be high (Koivikko *et al.* 1991). Thus, further research on antigen characterization of molds and quality control of allergy test extracts is urgently needed.

The estimates of mold allergy prevalence vary in different populations. Mold allergy is more common in children than in adults (Mygind, 1986). In selected populations like asthmatics in humid climate the prevalence of mold sensitivity may be as high as 70 % (O'Neil *et al.* 1988). In southwestern Finland, almost 30 % of asthmatic children had specific IgE-antibodies to molds or yeasts (Koivikko *et al.* 1991). In non-selected child materials, the prevalence of skin test positivity has been relatively low, 5 %, but there is higher risk for mold allergy among boys and children with previously diagnosed asthma or other atopic disease (Taskinen *et al.* 1999). In Canada, 12 % of outdoor workers and 5 % of controls had positive skin prick test to *Alternaria*-extract (Gautrin *et al.* 1994). In Denmark in an unselected adult population, the frequency of positive mold skin prick test was 3.2 % and for *Cladosporium* 1.5 % (Nielsen, N. H. *et al.* 1994). The outdoor concentrations of fungi depend on the season and weather conditions (Hjelmroos, 1993). In subarctic climate, the patients allergic to molds have most prominent symptoms in the spring and in the autumn when outdoor concentrations are usually high (Goldfarb, 1968).

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 have been shown in several countries and climates (Brunekreef, 1992; Waegemaekers *et al.* 1989; Dales *et al.* 1991). Swedish children (n>5000) had an odds ratio of 1.8 for asthma when living in apartments with water damage compared to nonexposed controls (Andrae *et al.* 1988). The association between chronic chest disease and indoor concentration of mold spores has also been found (van Wageningen *et al.* 1987). Also in some case-control studies, an association between asthma and mold exposure has been found (Burr *et al.* 1985; Hyvärinen *et al.* 1999). It is also obvious that mold exposure is a strong irritant factor and therefore worsens the symptoms of any pre-existing allergic disease like other nonspecific irritants do, such as tobacco smoke, traffic exhaust or cold air (Targonski *et al.* 1995; Dahl, Gerde 1994).

The occurrences of asthmatic symptoms show a clearer association with damp housing and mold exposure than doctor diagnosed asthma. In a number of studies, risk of asthmatic symptoms associated with mold exposure has been 2-4 times higher compared with reference groups both in children and adults (Brunekreef *et al.* 1989; Platt *et al.* 1989; Strachan, 1988; Burr *et al.* 1981). E.g. in a large Canadian material (n=12 000), the odds ratio for asthma was 1.5 and the OR for chest tightness was 1.6 in association with mold damages at home (Dekker *et al.* 1991).

It has been suggested that increased prevalence of allergy associated with home dampness would be rather due to mite allergy than to mold allergy because IgE-antibodies to mites and to molds are often found in same persons (Brunekreef *et al.* 1989). This may be true in moderate and humid climates where dampness and mites are present in the same apartments (Hart, Whitehead, 1990; Burr *et al.* 1980). However in colder subarctic climate e.g. in Finland, the house dust mites were equally rare in water damaged homes and in reference apartments during the winter (Hyvärinen *et al.* 1993). In a Dutch case control study of children with chronic respiratory symptoms, home dampness was associated with increased sensitization to dust mites and molds. The prevalence of IgE antibodies against mites was 36 % and against molds 9 % in symptomatic children. The respective prevalences in non-symptomatic children were 12 % and 0.1 % (2 cases). Only five children were sensitized to molds but not to mites (Verhoeff AP *et al.* 1995). The sensitization to mites in a Danish adult population was 14 % and to molds 3.2 % as measured with skin prick tests (Nielsen *et al.* 1994).

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. It has been calculated that alveolar deposition rate is highest in

those particles with aerodynamic size around 3 micrometers (Reponen, 1994). Molds for ex. *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 (Pepys, 1969; Terho *et al.* 1987)

Allergic alveolitis has traditionally been reported in occupational exposure situations and seldom in indoor air exposure. The most common form of alveolitis is farmer's lung. Other types of alveolitis caused by occupational exposure are found e.g. in sawmill workers, green house workers, mushroom workers and among exotic hobbies like pidgeon breeding etc. In indoor air exposure, only sporadic cases of alveolitis have been reported. A few cases of alveolitis caused by *Penicillium sp.* and yeasts like *Sporobolomyces* and *Rhodotorula* have been published (de Hoyos *et al.* 1993; Cockcroft *et al.* 1983; Park *et al.* 1994).

The distinction between humidifier fever and hypersensitivity pneumonitis is sometimes not quite clear and in the acute phase symptoms are quite similar. Suda and coworkers have reported cases of hypersensitivity pneumonitis associated with use of an ultrasonic humidifier. The patients had precipitins to *Cephalosporium* and *Candida*. Fungi and bacteria could be cultivated from the water (Suda *et al.* 1995). Also bacterial endotoxin and algae in humidifiers and cooling towers may cause fever and other chest symptoms related to humidifier fever (Edwards *et al.* 1977).

The differential diagnosis between alveolitis and organic dust toxic syndrome (ODTS) is also vague, and commonly accepted diagnostic criteria for ODTS have not been published. It is, however, a shared opinion of a number of researchers that in ODTS, reversible functional changes of the lung may occur and elevated cell counts may be found in bronchoalveolar lavage, but fibrosis is not detected either in chest x-ray or in lung biopsy. Symptoms in alveolitis and in ODTS are largely the same. Typical symptoms are fever or chills, malaise, dry cough, chest tightness, muscle and joint pains and in ODTS, also headache and symptoms of the urinary tract. Symptoms of ODTS seem to occur relatively often in mold exposure both in work places and in apartments. Both ODTS and alveolar inflammation may be under-diagnosed in indoor air exposure and long term follow-up studies of ODTS are lacking.

Chronic Bronchitis: Chronic bronchitis is defined as cough and upcoming phlegm for more than three months in two or more years. The association between microbial exposure and high prevalence of chronic bronchitis has been reported among nonsmoking farmers who have mold exposure related to their work (Terho *et al.* 1987). Similar preliminary results have been found between indoor air exposure and symptoms of chronic bronchitis. The increased risk of chronic bronchi-

tis associated with living in water-damaged buildings was statistically significant when adjusted for age, sex, atopy and smoking (Husman *et al.* 1993). In chronic bronchitis, bronchoalveolar lavage fluid contains increased proportions of neutrophilic cells thus differing the underlying immunological response from alveolitis (Balbi *et al.* 1994). The possible association between molds in indoor air and symptoms of chronic bronchitis needs further attention in future research. Chronic bronchitis occurs rarely in children and the prevalence increases in older age groups.

CONCLUSIONS

In the future, systematic intervention studies are needed which could show the effect of cessation or reduction of exposure after remedial measures in the building. Preliminary findings of follow-up studies are encouraging (Haverinen *et al.* 1999). Common respiratory infections and allergies have huge economical consequences and even small reduction in incidence rates may have considerable effect even in population level (Nguyen *et al.* 1998). The exact inflammatory and immunological processes behind the association between mold and allergy are still largely unknown. Further studies are needed on pathophysiological mechanisms of molds and other moisture related microbes as well as experimental research on human cells and animals. (Hirvonen *et al.* 1997; Hirvonen *et al.* 1999). Inflammatory processes of the respiratory system are subject to wide and vivid research at the moment. The evaluation of exposure and risk assessment of indoor air problems calls for better cooperation and coordination between several experts in research, occupational health service, labor protection, and environmental health surveillance (Husman, 1999).

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LABORATORY SIGNS OF SICKNESS DUE TO MOLD METABOLITES IN INDOOR ENVIRONMENT

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ABSTRACT

To verify that symptoms of ill health may follow exposure to mold metabolites, we tested 35 children (ages 10-14 yr) attending a school structurally repaired 0.5 yr earlier for mold problems. The *in vitro* leukocyte responses and IgG antibodies to molds were analyzed with respect to clinical status at the time of collecting blood samples, and to questionnaire filled 1 yr earlier. Subject groups were a) healthy controls, b) previously diagnosed asthmatics/allergics (=independent allergy, IA), c) those with both IA and "mold trouble" (=MT), d) those with MT. Higher levels of IgG antibodies and stronger TNF- α net production against molds associated significantly with allergy. Spontaneous lymphocyte proliferation in cell culture and eosinophilic leukotriene production were reduced by MT. IA appeared to increase the risk of "mold trouble" but the conditions were immunologically separable. The increased susceptibility to infections in "mold trouble" may result from abnormal Th-2 activity.

INDEX TERMS: Sick-building syndrome, Mold hypersensitivity diagnostics, Lymphocyte cultures, Cytokine secretion

INTRODUCTION

The association of moisture damaged buildings and clinical symptoms of occupants in general has been demonstrated in numerous studies, but difficulties are likely when the cause should be verified on diagnostic level for individuals applying compensation for disability e.g. from Social Security funds (Thörn, 1998). Diagnosis is validated by positive clinical challenge tests, with added evidence from hypersensitivity to microbes indicating moisture damage and found in the building in increased quantities. However, both skin tests and specific IgE antibodies are insensitive and unreliable diagnostic tools not only in our experience but also according to studies published elsewhere (Meyer *et al.* 1998; Malkin *et al.* 1998).

Very few publications deal with the production of inflammatory mediators by the mold-exposed individuals: Walinder *et al.* (2001) examined nasal lavage fluid for concentrations of eosinophil cationic protein (ECP), myeloperoxidase, albumin, and presence of eosinophils, and Purokivi *et al.* (2001) measured IL-1, IL-4, IL-6 and TNF- α in nasal lavage fluids and induced sputum samples (and ECP in the latter). With healthy and asthmatic/allergic subjects, some cytokines released in cultures of bronchial epithelial cells have been studied to evaluate responses to allergens or pathogenic microbes, and cytokine responses to various antigens have been studied in cultures of peripheral blood cells (reviewed by Robinson, 2000). The scales of responses vary considerably between individuals and test methods, and no laboratory test has proven superior to others in differential diagnosis of mold hypersensitivity. Accordingly, we measured some immunological variables to evaluate the Th-1/Th-2 balance in children attending a moisture damaged school.

METHODS

40 children (age 10-14 yr) were randomly selected from a rural school with moisture and mold problems to the occupants. Their parents filled in a large questionnaire on the family history of diseases, with an emphasis on the child assigned for the study. A year later, i.e. 6 months after a thorough structural repair of the school, 35 of the children consented to participate in the study. They were interviewed and examined clinically by a pediatrician to confirm the histories. None had signs of current infection, but irritation of skin and mucous membranes occurred in some. Only medication was bronchospasmolytics for the asthmatics.

Following the clinical examination blood samples were drawn. All tests were run in triplicate. Serum IgG antibodies (in-house EIA method) were tested with a panel of 11 microbial antigens, some commercial (ALK-Abelló Group, Horsholm, Denmark), some locally produced: *Aspergillus fumigatus*, *A. versicolor*, *Cephalosporium curtipes*, *Cladosporium cladosporioides*, *Fusarium solani*, *Stachybotrus chartatum*, *Geotrichum candidum*, *Penicillium brevicompactum*, *Rhodotorula rubra*, *Scopulariopsis brevicaulis* and *Trichoderma viride*. 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 median of the 11 OD-values was calculated.

Polymorphonuclear leukocyte cultures were stimulated with 2-3 selected mold antigen preparations, house dust and occasional other control allergen preparations (Pharmacia&Upjohn, Uppsala, Sweden); unstimulated control cultures were included. The 1-day culture supernatants were tested for secretion of eosinophil cationic protein (ECP) by UniCAP method (Pharmacia), and also for leukotriene (Ltr) secretion by CAST-ELISA method (Bühlmann Laboratories, Basel, Switzerland).

Lymphocyte cultures were stimulated with 3-4 mold antigen preparations and house dust antigen. The 1- and 5-day supernatants were tested for IFN- γ and TNF- α , IL-5, IL-6 and IL10 with commercial EIA kits from Genzyme (Cambridge, MA, U.S.A.) and CLB (Amsterdam, the Netherlands), as were the unstimulated control cultures. Of the latter, as well as of cultures with phyto-hemagglutinin (PHA; Difco, Detroit, Mich, U.S.A.), C3* incorporation (measured as CPM) was recorded. Cellular proliferation was counted microscopically from random third of samples of each culture type.

The subjects were classified in a) symptomless controls, b) those with previously diagnosed asthma or allergy (= independent allergy, IA) but no adverse effects at fungal exposure, c) those with both IA and clinical symptoms at school i.e. "mold trouble" (= MT), d) those with MT at school. Groups a) and b) were combined to analyze "mold resistance", b) and c) for conclusions about allergy, and c) and d) for "susceptibility to mold trouble". The grouping was based on school health records and filled-in questionnaires (provocation phase) with the interview/examination coinciding with blood sampling (remedial phase). The grouping and laboratory work were kept separate until statistical analysis. For each individual, mean stimulated secretion of each metabolite was recorded and compared with the unstimulated secretion. Medians were calculated for subject groups a), b), c) and d), and also for the combined groups. The statistical analysis was performed with the SPSS for Windows 9.1 software (SPSS Inc., Chicago, Illinois, U.S.A.), using the Mann-Whitney U-test with two-tailed p-values.

RESULTS

Table 1 combines the results in which notable differences were found between subject categories as defined by remedial phase. Table 2 shows the same results when subject categories are based on questionnaires of the previous year.

Calculations for TNF- α , IL-5 and ECP are not shown as significant differences in figures between the groups were not observed. Each of the 35 subjects developed a good response to PHA stimulation (measured in CPM), but the average cell counts in cultures stimulated with fungal or control antigens were essentially equal with counts from unstimulated (i.e. spontaneous) cultures (not shown in Tables). "Mold trouble" decreased both spontaneous mitoses (combined groups c) and d) in Table 2, $p=.024$) and stimulated mitoses (same groups in Table 1, $p=.038$).

IgG antibodies were produced by all 35 subjects even though the individual patterns varied considerably. The production was significantly stronger in children with a history of allergy (groups b) and c) in both Tables), $p<.001$. Highest values in the entire material were against *A. fumigatus* and *S. chartatum* antigens. This may or may not suggest the source of mutual fungal provocation - which was not ana-

lyzed microbiologically by commission of the municipal authorities prior to the school repair.

Table 1. Medians of recorded results in immunological variables of school-children. Remedial phase, division to groups by recent history and current status.

Variable	Group a (12), Symptomless	Group b (7), Allergic	Group c (7) Allergic + mold trouble	Group d (9) Mold trouble
IgG antibodies vs. molds	131 (132)	158 (77)	192 (77)	90 (99)
Mitoses (CPM)				
- spontaneous	752 (12)	439 (7)	335 (7)	501 (9)
- stimulated	53 (37)	1198 (22)	342 (22)	593 (27)
IFN- γ				
- spontaneous	40 (11)	21 (7)	5 (7)	11 (9)
- stimulated	40 (33)	45 (21)	17 (21)	42 (27)
IL-6				
- spontaneous	9150 (12)	4620 (7)	5100 (7)	9060 (9)
- stimulated	8970 (36)	5940 (21)	4020 (21)	7080 (27)
IL-10				
- spontaneous	31 (12)	17 (7)	17 (7)	26 (9)
- stimulated	37 (35)	27 (21)	18 (20)	17 (26)
Ltr				
- spontaneous	510 (8)	570 (5)	440 (7)	311 (8)
- stimulated	620 (13)	680 (9)	380 (11)	725 (11)

Number of observations in parenthesis. Antibodies expressed in OD values; cytokines and Ltr in pg/ml. **Bold** type indicates significant differences.

The releases of Th-1 cytokines TNF- α and IFN- γ in combined mold-resistant groups exceeded (not significantly) those in susceptible groups in remedial phase, and the same difference flickered in spontaneous releases when classified by provocation phase. In the combined IA group b)+c) TNF- α net secretion (= stimulated minus spontaneous) was stronger than in non-allergic subjects ($p=.015$). The poor IFN- γ stimulation response of those symptomless in provocation phase, Table 2, differed almost significantly from that in MT group d) ($p=.027$).

The expression of Th-2 cytokines IL-5, IL-6 and IL-10 was inconsistent. Spontaneous secretion of IL-5 by the symptomless was marginally stronger ($p=.046$) in grouping by provocation phase. In the remedial phase allergy seems to suppress IL-6 secretion (for stimulated secretion, a)+d) vs. b)+c), $p=.069$) although the only statistically significant difference is between groups a) and c), $p=.017$. The same effect appears in Table 2 as the difference between groups c) and d), $p=.018$. When net secretion (stimulated minus spontaneous) of IL-6 is considered, those with mold trouble react stronger than the allergic (not significant, $p=.067$). Stimulated IL-10 secretion in the remedial phase was significantly weaker in subjects with MT; c)+d) vs. a)+b), $p=.006$.

Table 2. Medians of recorded results in immunological variables of school-children. Provocation phase, division to groups by questionnaires filled a year previously.

Variable	Group a (10), Symptomless	Group b (2), Allergic	Group c(12) Allergic + mold trouble	Group d (11) Mold trouble
IgG antibodies vs. molds	116 (110)	225 (22)	174 (132)	90 (121)
Mitoses (CPM)				
- spontaneous	752 (10)	2268 (2)	302 (12)	519 (11)
- stimulated	563 (31)	1899 (7)	371 (37)	593 (33)
IFN- γ				
- spontaneous	38 (10)	15 (2)	15 (12)	25 (10)
- stimulated	24 (30)	31 (6)	44 (36)	49 (30)
IL-6				
- spontaneous	6320 (10)	6435 (2)	4860 (12)	10800 (11)
- stimulated	6540 (30)	7635 (6)	5545 (36)	9600 (33)
IL-10				
- spontaneous	20 (10)	50 (2)	17 (12)	34 (11)
- stimulated	21 (29)	37 (6)	21 (35)	30 (32)
Ltr				
- spontaneous	920 (7)	560 (2)	490 (10)	300 (9)
- stimulated	800 (11)	550 (3)	540 (17)	450 (13)

Number of observations in parenthesis. Antibodies expressed in OD values; cytokines and Ltr in pg/ml. **Bold** type indicates significant differences.

We managed to carry out only a limited number of experiments to evaluate eosinophilic leukocyte responses. ECP secretion did not differ significantly between the groups, but MT suppressed Ltr secretion significantly when the provocation phase grouping was applied (Table 2, spontaneous c)+d) vs. a)+b), $p=.004$; stimulated c)+d) vs. a)+b), $p=.008$). The same trends in Table 1 are not significant.

DISCUSSION

Even though the reproducibility of internal reference samples was excellent in our laboratory, the quantities of mediators secreted by individuals of each group varied widely. We observed quantitative differences also between this series and adults examined for occupational “mold trouble”. Our resources did not allow a reference study of children in a school free of mold problems, but for this particular study design group a) met our requirements of local reference. No reference values from the literature are applicable to our results. Culturing conditions, antigen preparations and techniques to measure the outcome of individual tests are not internationally standardized. – The small number of subjects studied by us is an obvious weakness which limits the statistical significance of the results. Regardless, after many years of extended search for informative diagnostic tests we presume that a combination of some cytokine analyses will become useful to the clinician. None of the mediators under study differentiated categorically individuals with MT from those without. A pattern of several tests is needed before it is possible to conclude whether hyperreactivity to molds is responsible for the clinical symptoms of an individual.

According to the questionnaires 23 of the 35 children had MT, but six months after the school repair five MT+IA children had shifted to IA group and two MT subjects now considered themselves symptomless. In provocation phase 12 of 14 IA children had MT, in remedial phase 7 of the 14; of 21 non-IA children 9 had prolonged MT. Hence, individuals with atopic/allergic/asthmatic history appear to have enhanced susceptibility to MT. - Even though subjects with IA are susceptible to MT these two conditions differ immunologically: mitotic activity, secretion of IL-10 and Ltr were suppressed in MT but IL-6 secretion was intact or even enhanced. On the other hand, IL-6 secretion was low in IA, and these subjects were best producers of IgG antibodies to molds. - Although nasal lavage and induced sputum samples may reflect rapid changes in mediator secretion following mold exposure of subjects at risk (Purokivi *et al.* 2001), the observation of increased IL-6 production parallels with our study of anamnestic responses *in vitro* after reduction of exposure. IL-6 seems to be an anti-inflammatory cytokine which enhances the levels of at least some other anti-inflammatory cytokines (Steensberg *et al.* 2003) and inhibits TNF- α production.

Obvious aberrations did not occur in Th-1 cytokine production in our series. In IA also Th-2 cytokine responses were consistent with the expected profile: mold antigens were only non-specific stimulators. In groups of subjects with MT Th-2 cytokine profiles were aberrant. Especially the low levels of IL-10 agree with this assessment: the experimental design of Akdis *et al.* (1998) supported other observations on the inhibitory effect of IL-10 on both Th-1 and Th-2 type lymphocytes; the inconsistency of our results justifies the hypothesis of some malfunctioning Th-2 cell subgroup(s).

Movérare *et al.* (2000) stated that the Th-1/Th-2 balance in immune response is polarized towards Th-2 dominance when the IL-5/IFN- γ ratio is high. The ratio appeared to be low in our subjects with MT; even so, strong Th-1 activity was not observed. Since IL-5 is a cytokine with specific action in the development, priming and survival of eosinophils (Robinson, 2000), the low release of eosinophil-derived inflammatory mediators ECP and Ltr in our groups c) and d) was comprehensible.

There is convincing evidence that Th-2 type T-cell responses play a role in human atopic disease (Robinson, 2000), whereas Th-1 type responses are associated with delayed hypersensitivity and considered protective against infections especially by intracellular pathogens. Since individuals with “mold trouble” appear to have suppressed Th-1 cell function and aberrant Th-2 cell function, their increased susceptibility to infections seems to be a logical consequence.

CONCLUSION AND IMPLICATIONS

Atopic/allergic constitution predisposes but is not a prerequisite to sickness due to mold metabolites. These conditions are immunologically distinguishable so that diagnostic use of combined cytokine tests is feasible. In MT many Th-2 cell functions are suppressed and aberrant, which in turn restrains Th-1 functions.

ACKNOWLEDGEMENTS

The financial sponsorship of the Social Insurance Institution of Finland is gratefully acknowledged. The authors thank Anne-Mari Moilanen for skilful technical assistance.

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ASSESSING THE ALLERGIC POTENTIAL OF INDOOR AIR FUNGAL CONTAMINANTS

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ABSTRACT

Molds, an important indoor environmental contaminant, have been associated with asthma exacerbation. However, their contribution to allergic asthma induction is less certain. Our objective was to assess the allergic potential of fungal extracts: *Metarhizium anisopliae* (MACA) and *Stachybotrys chartarum* (SCE-1). BALB/c mice were exposed by involuntary aspiration (IA) to MACA, SCE-1, bovine serum albumin (negative control), or vehicle. Serum and bronchoalveolar lavage fluid (BALF) were assessed for IgE and other indicators at various times relative to the final IA. Whole-body plethysmography was used to assess pulmonary resistance and bronchoconstriction. Exposure to these fungal extracts caused an increase in parameters characteristic of allergic lung disease as well as non-specific inflammation. Respiratory exposure to either MACA or SCE-1 causes responses similar to those observed in human allergic lung disease.

INDEX TERMS: Allergic Asthma, Fungus/Mold, *Stachybotrys chartarum*, *Metarhizium anisopliae*

INTRODUCTION

The incidence of asthma, a complex chronic respiratory disorder characterized by intermittent airway constriction and airway hyperresponsiveness, has increased since 1960 (CDC, 2001). Children exhibiting the most severe manifestations of asthma usually have a clear allergic component; symptoms begin in early in life, and continue through adolescence and into adulthood. Therefore, >90% of adult asthmatics are allergic asthmatics (Holt *et al.* 1999). The cause (s) of this increased inci-

dence are unknown, but lifestyle and/or environment changes are implicated. The fact that children are now spending >90% could potentially be important.

Molds, an important indoor environmental contaminant, have been associated with exacerbation of asthma. However, their contribution to allergic asthma induction is less certain. A number of well-characterized protein allergens including common indoor air contaminants such as house dust mite and cockroach antigens have been shown to produce sensitization and respiratory allergic responses in humans, guinea pigs and mice. Additionally, they have been strongly associated with asthma morbidity. However, very few of the mold allergens have been characterized, despite their widespread distribution and potential importance in the induction and exacerbation of asthma.

We have been investigating two fungi with suspected potential to induce allergic lung disease and therefore asthma (Ward *et al.* 1998, Ward *et al.* 2000, Viana *et al.* 2002). *Metarhizium anisopliae* is a biopesticide used to control a variety of pests (Gillespie 1988), including cockroaches. *Stachybotrys chartarum* is a toxigenic mold that has been associated with adverse health effects in water damaged homes and building (Mahmoudi, Gershwin, 2000). For both fungi there is anecdotal and some published reports (Kaufman, Bellas, 1996; Hodgson *et al.* 1998) linking them to asthma. The potential exists for genetically predisposed individuals inhabiting buildings or homes contaminated with either of these or other fungi to be exposed to fungal aerosols of whole or fragmented spores and/or mycelium, and to develop or exacerbate allergic lung disease.

Our objectives are to develop methods to assess the allergic potential of fungal extracts. We have used a mouse model to assess immune and inflammatory responses as well as changes in respiratory function and pathology characteristic of allergic asthma to fungal extracts MACA (*M. anisopliae*) (Ward *et al.* 2000) and SCE-1 (*S. chartarum*) (Viana *et al.* 2002).

Of additional importance in understanding allergic responses is the identification and characterization of specific protein allergens. Although no amino acid sequence motifs specific for allergenicity have been identified thus far, in general, protein sensitizers are hydrophilic, heat and digestion stable and may have enzymatic activity.

METHODS

Animals: Fifty day-old female BALB/c mice (Charles River, Raleigh, NC) were group-housed in an environmentally controlled, American Association for Accreditation of Laboratory Animal Care-accredited vivarium. Sentinel mice were

monitored and were found to be free of parasites and pathogens. Mice were allowed to acclimate one week prior to the start of the experiment.

Fungal Antigen Preparation: Five *Stachybotrys chartarum* isolates originally obtained from residences in Cleveland, OH (Etzcel *et al.* 1998) were grown and extracted as previously described (Viana *et al.* 2002). *Metarhizium anisopliae* strain 1080, obtained from USDA-ARS Entomopathogenic Fungus Collection in Ithaca, NY, was grown and extracted as previously described (Ward *et al.* 1998).

Experimental Design: Mice were exposed by involuntary aspiration (IA) 4 times over a four-week period, as previously described in Ward *et al.* (2000), to MACA or SCE-1 in 50 µl total volume. Concurrently, control mice were IA-exposed, 4 times over a four-week period to either HBSS (vehicle control) or bovine serum albumin (BSA, negative control). Additionally, as a control for non-specific inflammation, mice were IA exposed to 3 doses of HBSS and a final single dose of MACA or SCE-1. BALF and sera were collected before the last IA (D0) and 1 and 3 days later.

Bronchoalveolar Lavage and Blood Collection and Analysis: Blood and BALF samples were collected as previously described (Ward *et al.* 1998). Total BALF cells were counted using a hemacytometer with viability assessed by Trypan blue dye exclusion. BALF cell cytology slides were stained with Wright-Giemsa (Fisher Scientific) on a Hema-Tek 2000 slide stainer (Miles, Inc., Elkhart, IN). Differential WBC counts were performed by counting 200 cells per slide (one slide per animal). For total IgE ELISA, the paired antibodies rat anti-mouse IgE (PharMingen, San Diego, CA) and biotinylated rat anti-mouse IgE (PharMingen) were used for capture and detection. For the IL-5 ELISA, the paired antibodies anti-mouse IL-5 (PharMingen) and biotin-anti-mouse rIL-5 (PharMingen) were used for capture and detection. Optical density was read at a wavelength of 650 nm. Softmax Pro® software (version 2.6.1, Molecular Devices Corp.) was used for data collection and conversion from optical density to protein concentrations.

Measurements of Airway Responsiveness: Antigen-specific immediate airway responsiveness was measured as previously described (Viana *et al.* 2002) immediately after each of the four exposures to fungal extract in unrestrained mice using whole body plethysmography (Buxco Electronics, Troy, NY). Briefly, baseline PenH measurements (a unitless parameter, which strongly correlates with lung resistance (Hamelmann *et al.* 1997)) for each animal were recorded for 10 minutes and averaged. Animals then received a fungal extract IA, and placed back in the chambers within five minutes of dosing. PenH readings were then monitored and averaged over a 1 hour post-instillation period. Airway responsiveness to methacholine (MCh) aerosol was determined by measurement of time-integrated changes in PenH in response to MCh on D1 and D3 after the final IA. After meas-

urement of baseline PenH for 5 minutes, either saline or MCh in doubling concentrations (4 mg/ml up to 64 mg/ml) as previously described (Viana *et al.* 2002).

Statistics: The data collected were analyzed using analysis of variance. Pairwise comparisons were performed as subtests of the overall model. In cases of comparing a control group to other groups, Dunnett's Test was used. Significance was attributed to $p < 0.05$. For multiple comparisons a modified Bonferroni Correction was used.

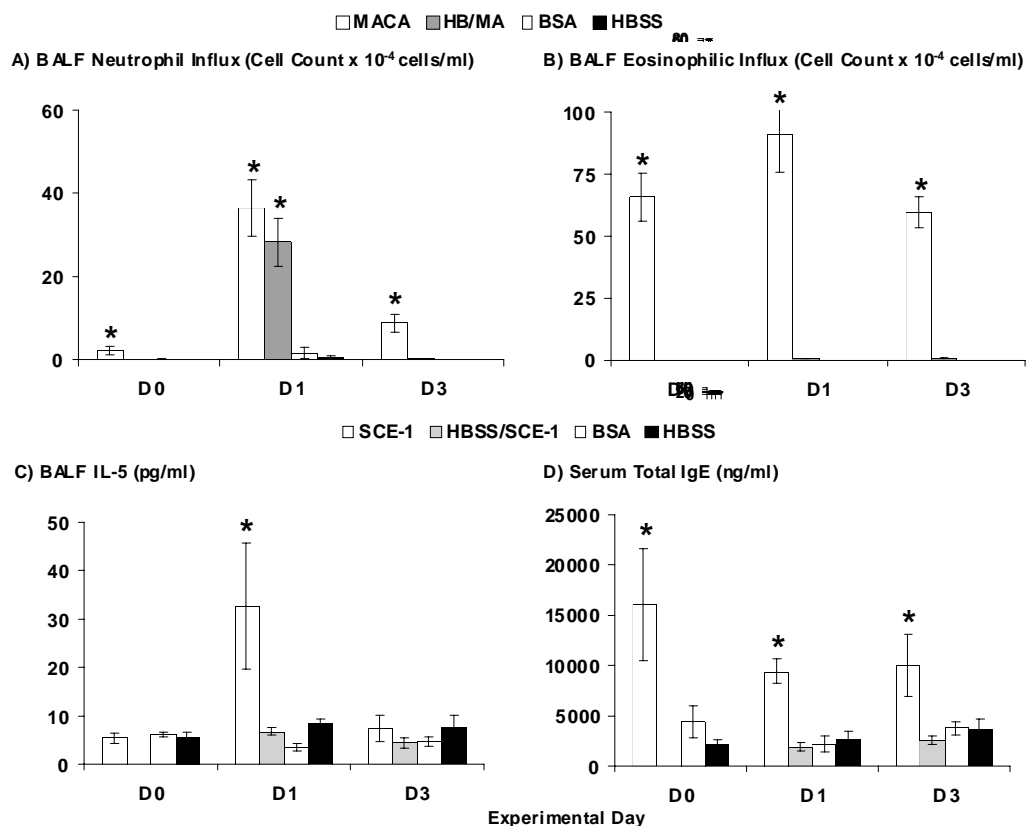
RESULTS

In BALF differential cell counts both SCE-1 and MACA caused a significant influx of neutrophils (characteristic of non-specific inflammation) (Fig. 1A (representative data shown in figure)) at D1. This influx of neutrophils persisted at reduced but significant levels in the MACA study through D3 but had resolved to control numbers by D3 for SCE-1. The inflammatory controls demonstrated a similar and significant pattern of neutrophilic influx. Mice sensitized to either fungal extract had significantly increased numbers of eosinophils (characteristic of allergic inflammation) (Fig. 1B) at all time points not seen in the non-specific inflammatory control. In both studies BALF from HBSS- and BSA-treated mice had very small numbers of neutrophils, and no eosinophils present at any time following the final IA.

IL-5 is the predominant cytokine involved in the production, activation, and localization of eosinophils (reviewed in Sanderson, 1992). Both MACA and SCE-1-treated mice demonstrated a significant increase in BALF IL-5 at D1 compared to controls, returning to control levels at D3 (Fig 1C).

Elevated serum total IgE levels are considered to be indicative of an allergic response. Mice treated 4 times with either SCE-1 or MACA demonstrated increased levels of total IgE in serum (Fig. 1D) at all time points including D0 (before the final IA) compared to HBSS-treated controls. Inflammatory control mice (one dose of fungal extract on D0) and those treated with BSA (negative control) had total IgE levels comparable to HBSS-treated controls.

Figure 1. A) BALF Neutrophil influx; B) Eosinophil influx from MACA study; C) BALF IL-5; D) Serum total IgE from SCE-1 study. *Significant difference from controls ($P < 0.05$). Error bars represent standard error of the mean; $n = 5-6$.



Antigen-specific immediate respiratory responses were measured for 1 hour following each aspiration exposure. Animals exposed to SCE-1 exhibited a small (approximately 10%) but statistically significant increase in PenH over baseline after the 2nd exposure. This increase continued with each subsequent exposure, rising to 470% over baseline after the 3rd exposure, and 560% above baseline after the 4th exposure. In the MACA study a small but significant increase (10%) increase in PenH over baseline was observed upon 1st exposure (D-28). The increase progressed with each of the next 3 exposures with percentages of 12%, 190%, and 403%, respectively. Control treatments did not cause increases in immediate responses over baseline at any exposure in either study.

Individuals suffering from allergic asthma demonstrate exaggerated bronchial airway hyperreactivity following exposure to the non-specific cholinergic agonist

methacholine (MCh). Therefore, airway hyperresponsiveness to increasing concentrations of nebulized MCh was assessed in all groups on days 1 and 3 following the 4th IA. Animals exposed to 4 doses of SCE-1 exhibit a significant increase in PenH at 8, 16, and 32 mg/ml of inhaled MCh on day 1 following the final IA, compared to both HBSS and other controls. This hyperresponsiveness persisted to D3, when SCE-1-treated mice showed an increased PenH following a 32 mg/ml MCh aerosol challenge (data not shown). Mice exposed to 4 doses of MACA exhibit a significant increase in PenH at 32 and 64 mg/ml of inhaled MCh on day 1 following the final IA, compared to both HBSS and other controls. This hyperresponsiveness persisted to D3. In both studies the negative control (BSA) and the inflammatory control mice responses were not statistically different from HBSS-treated mice at any MCh concentration on either D1 or D3.

DISCUSSION

Our results indicate mice sensitized and challenged by respiratory exposure to either SCE-1 or MACA developed both non-specific inflammatory responses as well as many of the responses associated with respiratory allergy and asthma. Specifically, mice receiving 4 doses of either fungal extract had increased serum IgE levels, significant influxes of lymphocytes (data not shown) and eosinophils, and increased levels of the Th2 cytokine IL-5. In contrast, animals exposed to only one dose of either fungal extract demonstrated non-specific inflammatory responses, including elevated BALF protein and LDH levels and neutrophilia, but did not have elevations in serum IgE levels, BALF IL-5, or eosinophilia. It has been suggested that these non-specific inflammatory reactions may be important in facilitating sensitization and the specific allergic responses by mobilizing pulmonary immune cells and/or increasing lung permeability.

Airway narrowing following exposure to an inhaled allergen is the primary physiological response in allergic asthma, and airway hyperresponsiveness to a non-specific stimulus, such as methacholine, is a diagnostic tool used in patients with suspected asthma. In these studies, mice previously sensitized to either SCE-1 or MACA had both increased immediate bronchoconstriction upon fungal extract challenge and increased airway hyperresponsiveness to the non-specific methacholine challenge. Animals exposed to a single dose of SCE-1 or MACA did not demonstrate immediate bronchoconstriction or increased airway hyperresponsiveness to a subsequent non-specific challenge, despite the presence of biochemical and pathological indices of pulmonary inflammation. This argues that the acute and methacholine-induced respiratory physiological responses we observed are allergen-induced, and not an artifact of a non-specific inflammatory response. Further, the biochemical and pathological responses, particularly the presence of eosinophils, were predictive of physiological changes in multiply exposed mice.

We have begun the task of identifying and characterizing IgE-inducing proteins in the fungal extracts. At least 4 IgE-inducing proteins have identified in the mycelia extract of *M. anisopliae*; however, there are some differences in allergen expression among the three extracts (mycelium, conidia, and inducible protease/chitinases) components. Furthermore, 8-10 putative allergens have been tentatively identified in Western blots of *Stachybotrys chartarum* extracts. While several allergens are comparably expressed among the 5 isolates under study, there are also several allergens that are differentially expressed among the isolates.

CONCLUSIONS

We have shown responses in mice exposed to either *Stachybotrys chartarum* or *M. anisopliae* extract that are consistent with human allergic airway disease. Therefore, we conclude that there are proteins in these aqueous extracts capable of inducing allergy in a susceptible population. The BALB/c mouse model provides a useful tool for hazard identification. This model may be useful to assess the potency of mold allergens relative to other indoor allergens as well as to help establish the relative importance of mold in the indoor environment. Ultimately, the identification of specific allergens will provide the tool needed to test humans for allergic antibodies to these microbes.

ACKNOWLEDGMENTS

Disclaimer: This paper has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflects the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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ALLEGED MERCURY POISONING SYMPTOMS IN SEWAGE TREATMENT WORKERS LIKELY LINKED TO BACTERIA, FUNGAL, AND ENDOTOXIN EXPOSURE

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ABSTRACT

Workers at a sewage treatment plant were diagnosed with mercury poisoning based on their self-reported symptoms. An investigation of exposures at the plant revealed negligible exposures to mercury vapor, CO, NO₂ or H₂S. The investigation did find elevated endotoxin levels consistent with elevated airborne concentrations of Gram-negative bacteria. Airborne endotoxin may have been responsible for the symptoms experienced by these workers.

INDEX TERMS: Endotoxin, occupational exposure, bioaerosol, Gram-negative bacteria, fungi

INTRODUCTION

Symptoms experienced by sewage treatment workers (five individuals) including nasal congestion, eye, nose, and throat irritation, headache, shortness of breath, nausea, diarrhea, light-headedness, fatigue were interpreted by a local physician, specializing in chelation therapy, as mercury poisoning. He supported this diagnosis based on the urinary mercury levels that were measured after a provocation test using DMPS (2,3-Dimercapto-1-propansulfonic acid) (i.e. subject #1, 21 µg/g of creatinine) and compared them to urine mercury levels measured in the general population under normal (non provocation) conditions (average 3.3 µg/g creatinine). It has been suggested however that unless urinary mercury levels are above 35µg/g creatinine there is no reason for concern (Klaassen, 1990). The physician nevertheless diagnosed mercury poisoning as a result of exposure to mercury vapor emanating from dental amalgams, known to accumulate at certain stages of the sewage treatment process. He further suggested that all workers should have all

amalgam fillings replaced with other material followed by extensive chelation therapy.

It has been well documented that workers in sewage treatment plants are exposed to a large variety of potentially toxic agents (Mulloy, 2001). The most predominant ones are bacteria and fungi, endotoxins, ammonia, and volatile organic compounds. Exposure to these agents have been linked to a high prevalence of respiratory problems including nasal congestion, eye nose, and throat irritation, headache, and shortness of breath (Nethercott, Holness, 1988; Lax *et al.* 1999). A Swedish study using a questionnaire (Thorn, Rylander, 2002), not only identified an increased risk of respiratory symptoms, but also an increased risk of gastrointestinal symptoms, joint pains, as well as central nervous system symptoms. Gastrointestinal symptoms including nausea and diarrhea have been related to endotoxin and viable fungal exposures (Ivens, 1999). Central nervous system symptoms have been related to the presence of organic solvents, which enter the sewage system from industrial waste (Kraut, 1988). The symptoms observed included light-headedness, fatigue, headache, increase in sleep requirement, and were considered consistent with solvent exposure.

High levels of exposure to endotoxins, from a variety of different Gram-negative bacteria are capable of provoking a weak immunogenic reaction, as well as producing a fever (pyrogenic). They can cause shock, induce weakness, diarrhea, inflammation, and intestinal hemorrhage. Endotoxins also interfere with the blood clotting reaction (factor XII, Hageman Factor)(Evans, 1998). Specific health outcomes from inhaled endotoxin exposures include: fatigue, mucosal irritation, malaise, cough, chest tightness, and acute airflow obstruction (Milton, 1999). Low level endotoxin exposures may be associated with asthma and other symptoms resembling Sick Building Syndrome (Teeuw *et al.* 1994, Michel *et al.* 1991).

It was our objective to identify, or eliminate, mercury as the source of health complaints from workers at this plant and its associated pumping station and, if mercury exposure was not present, to identify alternate exposures which could explain the observed symptoms.

METHODS

Airborne mercury concentrations were measured using SKC Carulite tubes (Hydar lot#2066) attached to personal air sampling pumps set at 0.2 liters/minute and carried in the respiratory zone of the persons being monitored. After exposure the tubes were sent to an analytical laboratory (ALS, Vancouver, BC) for mercury analysis using the British Columbia Workers' Compensation Board Method 1000.

Measurements for carbon monoxide (CO), oxygen (O₂), nitrogen dioxide (NO₂), and hydrogen sulfide (H₂S) were taken in areas throughout the plant (inside and outside) at random sampling times, several times a day for 5 consecutive days resulting in a total of 118 data points for each of the agents of interest. An Industrial Scientific TMX-412 direct reading gas monitor was used to determine concentrations of CO, O₂, and nitrogen dioxide (NO₂). H₂S concentrations were measured using a 1800 series direct reading Biosystems Toxilog monitor with an electrochemical sensor for this gas.

Measurements for the Gram-negative bacteria and fungi were done once a day, for 5 days, at selected locations at the work site identified in Table 1. Sampling took place in areas that were indicated by the employer and employees as those of concern.

Gram-negative bacteria and fungi were sampled for 10 minutes each at a rate of 28.3 liters per minute using the six-stage Andersen sampler. A ten minute sampling period was chosen to provide optimum results without overloading the plates. Eosin methylene blue agar was used for Gram-negative bacteria, and malt extract agar for fungal analysis. Bacterial plates were incubated for 48 hr at 35°C and fungal plates for 5 days at 21°C. Selected Gram-negative bacteria were identified using API 20E (BioMerieux). Fungal colonies were identified by microscopic examination of conidial structures at 400 x magnification using standard taxonomy texts. Field blanks were used to determine potential contamination during transport and storage of the samples.

Both personal and area samples were taken on each of the five day sampling periods for endotoxin. Area sites were chosen based on where workers would spend most of their time. These locations coincided with those areas which were also monitored for gases. All personal samples were placed within the breathing zone of the wearer and worn throughout their shift. As with the bacteria and fungi sampling, field blanks for each day were used.

Endotoxin was sampled using 7-hole samplers equipped with glass fiber filters using personal sampling pumps. The pumps were calibrated both pre and post sampling at a flow rate of ~2 liters per minute. Personal samples were taken when individuals were in sites listed in Table 1. The samples were analyzed for endotoxin using a kinetic *Limulus* amoebocyte lysate assay (BioWhittaker, Kinetic QCL) and read on a 96-well microplate reader (SpectroMax, Molecular Devices).

The individuals with complaints were re-evaluated for physical signs and symptoms by a qualified occupational health physician.

Table 1. Airborne bacterial, fungal, and endotoxin levels in the sewage treatment plant (STP) and pumping station (PS).

Location	Fungi						Bacteria			Endotoxin area EU/m ³	Endotoxin personal EU/m ³	
	CFU/m ³	Aureobasidium	Cladosporium	Penicillium	Sterile mycelia	Yeast	Other *	CFU/m ³	Lactose-fermenting Gram negative rods			Non-fermenting Gram negative rods
Treated sludge loading area (STP)	537	0.9	65	17	6	11	--	8		100	0.58	
Gravity belt thickener (STP)	1353	5	12	7	2	73	1	3283	8	92	21	4.8
Above aeration tank (STP)	985	0.7	32	35	15	29		491	7	93	3.78	8
Outdoor upwind (STP)	15370	0.4	48	19	18	13	1	4		100		
Office (STP)											< 0.05	<0.05
Pumping station (PS)	31537	0.8	4	5	0.8	90	0.1	5954	13	87	32	51
Outdoor upwind (PS)	2332	0.8	69	12	2	13	3	8		100		

* Includes Botrytis, Epicoccum, Geotrichum, and Mucor.

RESULTS

Airborne mercury measurements inside the facility indicated extremely low concentrations ($< 0.005 \mu\text{g}/\text{m}^3$), even in areas where sewage sludge was heated and open to the atmosphere. Additional measurements during the opening and cleaning of normally closed pipes did not indicate a mercury vapor problem but did indicate the presence of mercury in the scale inside the pipe.

Of the 118 determinations that were made for each of the gases monitored, none were above the TWA (Time Weighted Average NO_2 3 ppm; CO 25 ppm; H_2S 10 ppm)(ACGIH, 2002).

Oxygen levels during the 5 days of monitoring were generally at the ambient 21 % level range.

The overall results for bacteria, fungi and endotoxin have been summarized in Table 1. The Andersen sampler separated the fungal particulate into aerodynamic size ranges relevant to lung deposition. As identified in table 1, approximately 5% of the viable fungi cultured were small enough (e.g. $\geq 3.3 \mu\text{m}$) to enter the deep lung.

Between 20 to 50 % of the bacterial colonies were recovered from the last three stages of the Andersen (e.g. $\leq 3.3 \mu\text{m}$).

The independent medical assessments of each of the workers by a qualified physician and specialist in occupational medicine did not support mercury poisoning as a diagnosis. His findings did support the signs and symptoms that are associated with endotoxin exposure.

DISCUSSION

Although none of the environmental readings were of any concern, some of the elevated levels did identify areas of ventilation inadequacy resulting in local air stagnation. This was identified by elevated levels (2ppm) of H_2S which were well above background (0.1 ppm) in these areas. The TWA for H_2S of 10 ppm was never exceeded.

Somewhat elevated levels (5ppm) above background (< 1 ppm) of CO were measured inside the building at times when outside upwind levels were also elevated indicating an external source of this gas. The likely source of CO fluctuations was from car exhaust entering the ventilation system from cars on the parking lot and possibly from cars on an adjacent highway.

Occasionally levels between 20 and 21% oxygen were encountered. These slightly lower levels were usually measured in poorly ventilated areas and occasionally cor-

responded to elevated hydrogen sulfide levels. Although oxygen levels were always well above the 18% oxygen limit for asphyxiation, it did illustrate the fact that workers in these areas should always ensure the presence of an adequate fresh air supply.

Nitrogen dioxide levels were consistently below the 0.4 ppm levels. Higher levels around 1ppm were encountered in areas where CO was also occasionally elevated. At no time were levels higher than the 3 ppm TWA measured.

The ubiquitous environmental fungi *Penicillium* and *Cladosporium* constituted the majority of the fungi found. Very high levels of these fungi were found upwind from the sewage treatment plant. This was anticipated as this area was densely colonized with blackberry bushes, which were loaded with rotting blackberries at the time of monitoring. The type of organisms encountered inside the treatment plant were quite different and a high percentage of *Aureobasidium*, yeast, sterile mycelium, *Mucor* and *Geotrichum* were identified, indicating internal sources of these organisms.

The vast majority of the bacteria found in the samples were environmental organisms i.e. Gram-negative, non-fermenting, oxidase positive rods. These included *Pseudomonas sp.* and *Erwinia*. Enteric-origin bacteria were present as well. These included *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*, and *Citrobacter*. These organisms also have a relatively high endotoxic potential, and although they are not usually pathogenic, they are considered to be potential pathogens.

Ambient background levels of endotoxin were well below the detection limit of 0.05 EU. Those individuals working in the central office area of the sewage treatment plant were not exposed to levels above 0.05 EU/m³. Individuals who were working in the plant itself however experienced exposures to endotoxin well above these ambient levels. The highest level being measured was associated with one system operator which reached 1000 times background i.e. 50.8 EU/m³. Most of the time worker exposure was found to be around 100 times background levels.

As anticipated, those areas where the highest levels of endotoxin were measured also indicated the highest concentration of Gram-negative bacteria. Although some of the endotoxin exposures were elevated well above ambient levels they should be interpreted with care. The highest level measured was at the 50 EU/m³ limit recommended by the Dutch expert committee. This level is, on the other hand, well below the 200 EU/m³ accepted by the European community as the occupational exposure limit. Milton (1996, 1999) has proposed a relative threshold limit for endotoxin of 10 times (Milton 1996, 1999) and 30 times background, depending on the presence or absence of symptoms. Endotoxin values in this workplace exceeded both relative thresholds.

Individuals working in those areas where elevated levels of bacteria, fungi, and endotoxins were encountered, specifically the pumping station, were advised that they were at greater risk of dermatitis, skin infections, and symptoms of endotoxin exposure. Good personal hygiene was therefore recommended. This recommendation was even more important since live indicator organisms were present not only in the pumping station, as expected, but also in the plant itself at locations after the pasteurization process, indicating that this was not effective and/or there was another uncontrolled source of contamination in the sewage plant.

CONCLUSIONS

Although mercury was detected inside normally sealed pipes and identified as a potential source of exposure, it did not result in any inhalation exposure. The symptoms that were experienced by the workers were consistent with those that have been reported with exposures to similar levels of bacteria, fungi and endotoxin. Improved engineering controls and ventilation were recommended along with better personal hygiene practices and respiratory protection. A reliable monitoring program to ensure their effectiveness was also recommended.

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EPIDEMIOLOGICAL INVESTIGATION OF A MOLD-CONTAMINATED “SICK” BUILDING

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ABSTRACT

An innovative, software-based occupant health survey instrument and multivariate statistical analysis based on a retrospective cohort methodology was used to investigate occupant illness in a water-damaged, “sick” office building. Based on a case definition conservatively derived through principal components analysis, a highly statistically significant incidence and attributable risk of respiratory, neurocognitive, and constitutional symptoms was measured among occupants in comparison with those in a control building. Logistic regression analysis demonstrated that the collective symptoms were not explained by such building-related illnesses as asthma or hypersensitivity pneumonitis, nor by pre-existing medical conditions, smoking, or residential environmental factors. Cases were uniformly distributed among occupants throughout the building. The epidemiological findings were consistent with the known health effects of indoor mold exposure, and were not explained by either an allergic or infectious mechanism of disease.

INDEX TERMS: Sick building syndrome, Epidemiology, Building related symptoms, Indoor air quality, Mold

INTRODUCTION

In the past 10 years, exposure to certain types of mold in water-damaged buildings has been increasingly implicated as the cause of sick building syndrome (SBS). Epidemiological investigation of occupants within a given building or workplace population is a well-established scientific method used to identify and measure the nature, distribution, and cause of occupational or environmental illness. In its publication, *Bioaerosols: Assessment and Control*, the American Conference of

Governmental Industrial Hygienists underscored the importance of epidemiological investigation of occupant/indoor air quality (IAQ)-related health problems as a means to “clarify whether there is a building-related problem and, if so, its nature as well as possible means for resolution,” and emphasized the need for improved study design and statistical analysis for conducting an inherently complex investigation (ACGIH, 1999). However, most published, paper-based occupant health questionnaires and analysis methods have suffered from significant methodological limitations in their ability to define and measure occupant symptomatology as a multi-organ syndrome, and control for confounding variables (AIHA, 1996). The study herein employed a new epidemiological instrument and approach to analysis of complex occupant symptoms in a water-damaged “sick building” that had predominantly hidden mold contamination.

METHODS

Study and control buildings: The study building, located in northern Nevada, was a leased, 1-story, *ca.* 10,000 square foot office facility with central heating and air conditioning that housed a government agency since the late 1970's. Ongoing, unexplained employee health complaints among the 110 full-time government employees and an associated increase in employee absences and medical treatment occurred over a period of years. Traditional IAQ measures (CO₂, CO, temperature, relative humidity) were normal, and the ventilation system was found to be operating within acceptable parameters. The building was ultimately deemed to be a “sick building.” An IAQ investigation in 2001 revealed a longstanding history of un-repaired roof leaks resulting in stained suspended ceiling tiles and dripping water from the ceiling onto desks and floor space throughout the building. It was learned that in 1998, two interior walls in separate corners of the building had been discovered to be saturated with water. Occupants had observed musty odors in these areas since then. The landlord had responded by painting over these walls. In 2001, numerous bulk culture and tape samples of water-damaged gypsum board surfaces and on HVAC return filters demonstrated *Stachybotrys chartarum*, *Aspergillus versicolor*, *A. niger*, and/or *Penicillium* hyphae and spores. Non-viable (Air-O-Cell) air samples were dominated by *Aspergillus*/*Penicillium* spores.

The control building was a one-story, approximately 20,000 square foot, centrally heated and cooled, county government facility located in southern Nevada, constructed in 1995. There were 98 full-time occupants. The building had a history of a few minor plumbing leaks which had been promptly repaired, but otherwise did not have a history of occupant health complaints, investigations, or IAQ problems including visible mold contamination. A formal visual inspection and environmental sampling of the entire building identified some old water-stained ceiling tiles in various areas of the building, but no associated surface mold growth or odors.

Extensive air (viable and non-viable) and settled dust carpet samples demonstrated no atypical mold contaminant taxa.

Survey participants (respondents) in the study building were full-time occupants who were recruited by the employer at the beginning of the environmental and occupant health investigation. Control building respondents were recruited as part of an unrelated epidemiological study in 2001. All respondents provided written informed consent to participate in the survey.

Survey instrument and methodology: The epidemiological study was of a retrospective cohort design, with a non-problem building serving as the control. Occupant health data was collected using the EpIAQ® software (Verdi Technology Associates, LLC, Verdi, NV, U.S.A.), a proprietary, entirely computer-based occupant health survey instrument that was developed based upon existing, widely-accepted and utilized methods of epidemiological investigation of building-related illness/symptoms (NIOSH, 1996; Quinlan, 1989). All questions beyond demographics were closed-ended, with either yes/no or multiple choice responses. The design, content, and interaction features of this PC-based survey instrument obviated many of the methodological limitations of currently available and/or previously published, paper-based SBS/BRI/IAQ surveys. Specifically, major sources of recall, response, interviewer, and misclassification bias were eliminated or minimized by ensuring data collection reliability and enforcing completion of all survey questions with unequivocal responses; providing multi-level question complexity using conditional logic to query about symptom frequency and effect when away from the building; individualizing each respondent's symptom questions by temporal and geographic parameters related to the building's construction, occupancy, and layout; addressing potentially confounding pre-existing medical conditions, habits, and non-occupational environmental factors; measuring internal validation of response consistency and reliability; and providing program security features to ensure medical confidentiality, privacy and data integrity. Internal validation measures for consistency and plausibility of responses were incorporated into the survey design.

The SAS statistical analysis software (v. 8.1, SAS Institute, Cary, NC, U.S.A.) was used for data analysis. All tests were conducted at a significance (α) level of 0.05 (two-tailed), and reported as either *p* values or odds ratios. Demographic parameters were compared using t-tests for continuous variables, and Mann-Whitney or chi-square (χ^2) tests for non-continuous variables. Principal components analysis (PCA, also known as factor analysis), a multivariate statistical methodology that analyzes the interrelationship of variables in complex data (Johnson, 1982), was employed to measure the inter-relationship between 61 primary symptom questions and, for each affirmative response, 5 follow-up sub-questions related to

symptom frequency and building-relatedness among all responses from both buildings. Multivariate analysis of variance (MANOVA) on the first six principal components was then performed with respect to building, and reported as an F-statistic (Wilks Λ). If this overall building difference was statistically significant, a univariate ANOVA on each principal component was conducted. The first principal component (largest eigenvalue and a statistically significant ANOVA) was selected to conservatively define a case of “building-related illness” (“BRI”) based on the 95th percentile of control building symptom factor scores. The incidence of “BRI” in the study building relative to the pre-defined, conservatively assumed incidence (5%) in the control building was calculated as an attributable risk and attributable risk percentage, i.e., the proportion of illness among study (“exposed”) building respondents that is attributable to the building.

The symptoms most and least associated with the PCA-derived case definition of “BRI” were ranked according to their principal-component-derived eigenvector coefficients (i.e., factor loadings). The “BRI” case definition was compared to clinical case definitions of traditionally accepted building-related illnesses including asthma, hypersensitivity pneumonitis, and somatization disorder (as a surrogate for “mass hysteria”) to determine whether the collective symptomatology was clinically consistent with one or more of these disorders. The effect(s) of potentially confounding variables, namely pre-existing medical conditions, current and past smoking, and residential environmental factors, was analyzed using logistic regression analysis. Temporal and geographic variables and their effect on risk for and distribution of “BRI” was analyzed by logistic regression according to office/work area location; relative time spent in the office, building, or outside the building; and duration of occupancy. Finally, several forms of internal validation for symptom number outliers and response consistency were compared between buildings using χ^2 tests.

RESULTS

52% of the study building occupants completed the EpIAQ® survey. Four (4) incomplete surveys were eliminated from the analysis, and no duplicate surveys were identified. Participation in the control building was 69%, with no incomplete or duplicate surveys identified. Demographic comparison of the two building populations showed no statistically significant differences with regard to distribution of gender, age, smoking (current and past), residential factors, and workplace indoor exposure measures. The frequency of various pre-existing conditions, including asthma, allergic rhinitis/hay fever, depression, and chronic sinusitis was also similar, except for a slightly greater prevalence of obesity in the control building ($p = 0.05$). The prevalence of common (and uncommon) pre-existing medical condi-

tions in both populations was comparable and consistent with national prevalence figures.

MANOVA on all six of the principal components with respect to building demonstrated a highly significant difference (Wilks $\Lambda = 11.00$, $p < 0.0001$) in occupant symptomatology between the two buildings. The first principal component explained a substantial part of the variability in symptoms among subjects, with a highly significant building difference ($F = 34.35$, $p < 0.0001$), and was selected as the basis for the case definition of “BRI.” The difference in the mean number of symptoms among respondents in the study (22) vs. control (8) buildings was also highly significant ($t = 7.26$, $p < 0.0001$). 49% of the respondents of the study building met the case definition of “BRI” versus the pre-defined 6% (rounded off) in the control building, the difference of which was highly statistically significant ($\chi^2 = 24.82$, $p < 0.0001$). The attributable risk between study and control buildings was 43%, and the attributable risk percentage was 88%. Neurocognitive (forgetfulness, short-term memory loss) and respiratory symptoms (shortness of breath, shortness of breath with exertion) contributed most significantly to the case definition of “BRI,” i.e., symptoms with the highest eigenvector coefficients. Other symptoms also highly associated with “BRI” included difficulty concentrating, mood irritability, unexplained sadness, loss of interest in sex, and unusual fatigue. “Dummy” symptoms unrelated to IAQ comprised most of the least associated symptoms. None (0%) of the “BRI” cases in the study building was comprised of a combination of symptoms consistent with or explained by asthma, hypersensitivity pneumonitis, upper respiratory tract infections, or somatization.

Logistic regression analyses on numerous pre-existing immunocompromising diseases demonstrated no predictive relationship to or increased the risk for “BRI,” except for “intravenous antibiotic treatment” ($p = 0.02$) and treatment with oral corticosteroids (Prednisone, Medrol, $p = 0.09$). Among the plausibly building-aggravated upper or lower respiratory tract allergic or infectious diseases (asthma, hay fever/seasonal allergies, or chronic sinusitis), none (0%) had a statistically significant association with “BRI.” For pre-existing psychiatric conditions, depression ($p = 0.03$) and panic attacks ($p = 0.02$) were significantly associated with risk for “BRI.” Current ($p = 0.36$) and past ($p = 0.64$) cigarette smoking also had no statistical association with risk of “BRI.” Residential exposure factors including second-hand smoke, pets, and water damage in the home, did not explain or increase risk for “BRI.” Respondents in the study building were significantly more likely to seek medical attention for building-related symptoms than those in the control building ($p = 0.0009$), and within the study building itself, respondents with “BRI” had an increased likelihood ($p = 0.0003$) of seeking medical attention versus those who did not have “BRI.” The frequency of self-reported or misdiagnosed conditions of “continuous or unusually severe allergies” ($p = 0.0003$) and “frequent head colds

and/or respiratory infections” ($p < 0.0001$) was significantly greater among occupants with “BRI,” though the prevalence of these specific disorders as pre-existing conditions was similar between buildings. The geographic distribution of “BRI” within the study building was uniform ($p = 0.99$). Logistic regression analysis on relative time spent in the office/work area ($p = 0.86$), total time spent inside the study building ($p = 0.96$), and duration of occupancy ($p = 0.92$) demonstrated no statistical association with risk for “BRI.”

Internal validation measures demonstrated that all of the “high symptom number” (95th percentile) respondents were from the study building ($\chi^2 = 5.49, p = 0.0192$), whereas 91% of the asymptomatic, or “low symptom number” (5th percentile) respondents were from the control building ($\chi^2 = 9.75, p = 0.0018$). The frequency of inconsistent responses to validation questions and redundant questions was comparable between buildings ($\chi^2 = 0.051, p = 0.82$).

DISCUSSION

Occupants of the study building experienced a significant incidence (attributable risk and attributable risk percentage) of building-related, upper and/or lower respiratory tract and neurocognitive symptoms. A consistent, single disease entity among symptomatic occupants was demonstrated by the collective findings of a relatively high incidence of symptoms attributable to the study building, the prominent neurocognitive/non-respiratory symptoms statistically associated with the case definition through multivariate analysis, a uniform distribution of symptomatic respondents throughout the building, a lack of cumulative or temporally-related effects of building occupancy, and a strong association of symptoms with self/mis-diagnoses of “allergies,” “frequent upper respiratory tract infections,” and treatments with antibiotics and corticosteroids. The approach of analyzing symptoms collectively and using PCA to develop a case definition of a symptom complex (syndrome) in comparison with a control building enabled this epidemiological method to distinguish that the building-related health effects were not plausibly explained by, or simply a variant of an allergic or otherwise immunologically-mediated, building-related illnesses such as asthma, hypersensitivity pneumonitis, or allergic rhinitis—diseases which a number of researchers and reviewers have concluded or attempted to show by relying upon methods that have analyzed symptoms in isolated manner using crude summary statistics and arbitrarily created, symptom category-based definitions solely among study building occupants (Kolstad, 2002, ACOEM, 2002; Hodgson, 1998). Occupants with a variety of pre-existing immunological and respiratory disorders, as well as past and present cigarette smokers, were also not at increased risk of symptomatology consistent with respiratory tract or other infectious disease, though symptoms were self/misdiagnosed as such—a finding which also contradicts a commonly espoused but scientif-

ically unsupported risk dictum for mold-contaminated buildings (NYCDOH, 1999). Occupant illness was not explained by other carefully sought, potentially confounding medical or environmental variables, “mass hysteria,” disease misclassification, collection bias, or chance. The major limitation of the study was the study building participation rate which, while consistent with other published SBS studies (Kolstad, 2002), was not sufficient to generalize the findings to the entire building population with statistical confidence.

CONCLUSIONS AND IMPLICATIONS

The epidemiological findings in this mold-contaminated, “sick” building are consistent with the health effects that have been described for certain taxa of toxigenic fungi that grow on certain water-damaged building materials and disseminate as spores into the occupied spaces of buildings (IICRC, 2003). In contrast to the commonly propounded theory that “non-specific” building-related symptoms do not represent a single medical disorder (Menzies, 1997), the principal symptoms measured in this “sick” building, as well as their distribution and building-relatedness, were collectively consistent with “building-related symptoms” (ACGIH, 1999), “building-related illness arising from microbial contamination of building materials caused by condensation and leaks” (AIHA, 1996), “non-infectious fungal indoor exposure syndrome” (NIFIES; Craner, 1999), and “fungal syndrome” (Johanning, 1999), each of which is clinically indistinguishable from SBS. The uniform distribution of cases throughout the building also contradicts the presumption that exposure to mold spores (and spore by-products) is confined only to localized areas where visible mold is found (NYCDOH, 1999), and underscores the importance of identifying “hidden” mold contamination (AIHA, 1996). Although the specific biochemical mechanism(s) and dose-response relationship by which these molds cause their effects remains speculative, these epidemiological findings point strongly toward a unifying toxicological mechanism (IICRC 2003). While occupant symptomatology has been the principal health endpoint which all epidemiological methodologies in the SBS/BRI/IAQ research field have focused upon, this new, more robust epidemiological instrument and approach described herein represents an advancement in scientific understanding of a complex occupational and environmental disease syndrome and its relationship to particular indoor environmental conditions and contaminants. Further epidemiological research using this approach will provide direction and support for clinical research into pathophysiological responses, toxicological mechanisms, and routes of exposure in occupants of “sick” buildings.

ACKNOWLEDGMENTS

Funding for this study was supplied through workers' compensation insurance and risk management funds as part of the investigation of the subject building.

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SYMPTOMATIC IMPROVEMENT AFTER CESSATION OF MOLD EXPOSURE: CLINICAL EXPERIENCE IN ENVIRONMENTAL AND OCCUPATIONAL HEALTH

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BACKGROUND/INTRODUCTION

Exposure to indoor mold is a growing concern to the public as well as health professionals, documented by increasing news coverage of the “toxic effects” of “killer molds” in schools, homes, and other buildings (Belkin, 2001; EPA, 2001; NIOSH, 2001; American Academy of Pediatrics, 1998). Mold spores are ubiquitous (indoors and out). With an appropriate substrate and moisture, mold will proliferate indoors. Occupants of mold-contaminated structures are potentially exposed to a number of mold-related agents, including antigens, microbial volatile organic chemicals (MVOCs) and mycotoxins (American Journal of Industrial Medicine, 2003, 43; Stark, 2000;).

Possible mechanisms for the health effects reported in association with mold exposure include allergy, infection, irritation or toxicity. Allergy is the most generally accepted pathophysiological explanation for mold-related symptoms. 10% of Americans are reported to have allergic reactions to fungal antigens of which about half, or 5%, are thought to have clinical illness related to mold exposure. (ACOEM, 2003) Several case reports cite an increase in respiratory symptoms and increased asthma in adults and children in damp buildings with visible mold. (Seuri *et al.* 2000; Su, Wu, Lin, 2001; Jaakkola, M *et al.* 2002). Additionally, non-specific symptoms of mucous membrane irritation, fatigue, headache, nausea, difficulty concentrating, and other vague complaints have been reported in buildings with known mold exposure (Johanning *et al.* 1999; Ebbehøj *et al.* 2002; Cooley *et al.* 1998;). The pathophysiological mechanism for these symptoms is not clear, but it has been hypothesized that MVOCs or mycotoxins may be causative agents. Fungal infections are not thought to comprise a significant risk in healthy people. To date, studies have not clearly demonstrated a causal relationship between mold exposure and non-allergic health effects (Ammann, 2000).

The objective of this clinical review was to describe the nature of mold related complaints seen in a general occupational medicine practice and to determine whether intervention (moving away or remediating the mold condition) resulted in improvement in medical symptoms and patients' overall assessment of their own health. This review addressed both classic allergic and respiratory symptoms as well as non-specific symptoms where the relationship between mold and symptoms is not well documented in the medical literature.

METHODS

A retrospective analysis was conducted on patients seen at the EOHSI Clinical Center, a referral center for a wide variety of environmental and occupational health problems, during the period between 1991-2002. 40 patients were identified from our clinical database as having had health complaints related to mold exposure. Medical records were obtained and reviewed. Demographics, exposure characteristics, presenting symptoms, presence of asthma, documented allergy testing and patient assessment of overall health status (poor, fair, good, excellent) at the time of initial visit were extracted. All of the patients included in the study had documented mold exposures as confirmed by industrial hygienists, environmental consulting firms, or photographic evidence of mold contamination.

Self-reported symptoms were recorded from a standard written checklist (review of systems) collected on all clinical center patients at the time of initial examination. Patients were asked if their symptoms were related to occupational exposures or exposures in their homes. Respiratory symptoms were divided into upper and lower respiratory. Upper respiratory symptoms included post-nasal drip (PND), sinus and eye complaints, nasal congestion, rhinorrhea, and sore throat. Lower respiratory symptoms were defined as cough, shortness of breath, wheezing, and chest tightness. The remainder of the symptoms described by the patients was considered non-specific for the purpose of this analysis. Because of the large number of patients reporting headache and fatigue, these two symptoms were individually scored. The other symptoms were placed in three groups. Cognitive and psychological complaints were defined as lack of concentration, decreased memory, sleep problems, irritability, and depression. Neurologic symptoms included muscle weakness, numbness, lightheadedness, and dizziness. Gastrointestinal symptoms included abdominal pain, nausea, and diarrhea. The patient's overall assessment of their health (poor, fair, good, or excellent) was also recorded at the time of their initial visit.

At follow-up, a scripted telephone questionnaire was administered to patients who were previously evaluated in the clinical center for mold exposure and possible health issues. These interviews took place between August 2002 and February

2003, which represented a time period of 6 months to two years after the initial contact with the majority of patients. Two (2) patients were contacted who were seen ten (10) years prior to the interview. Verbal informed consent was obtained for the interview and the study was approved by the UMDNJ-RWJMS IRB. Patients were assessed regarding changes in their medical symptoms and their overall assessment of their own health. They were also asked about presence or absence of mold in their location of concern (home or work), and whether they left that environment, the mold exposure had been remediated, or whether the exposure was essentially unchanged from the time of original examination.

We assessed the following parameters: 1)-Change in respiratory (upper and lower) symptoms from initial visit to follow-up interview in both subjects who believed they eliminated their mold exposure and those who did not change their exposure. 2)-Change in non-specific symptom categories (headache, fatigue, cognitive, neurological, and GI. 3)-Change in number of total symptoms (both respiratory and non-specific). 4)-Change in self-reported health assessment (i.e. poor, good, excellent vs. no change in reported patient reported symptoms). The interviewer asked the patient to assess his or her own overall health in a similar manner to their original questionnaire.

RESULTS

40 patients were identified between the years from 1991-2002 with exposure to mold. 27 (67.5%) patients were female. The mean age at the time of initial clinical visit was 34.9. 13 (32.5%) reported exposure at home and 27 (67.5%) reported exposure at work. 11 (27.5%) reported a history of asthma currently being treated at initial examination.

Review of the patient symptoms reported at their initial clinic visit found that 23 (57.5%) reported headaches, 28 (70%) reported fatigue, 32 (80%) reported cognitive/ psychological complaints, 34 (85%) reported neurological complaints, 23 (57.5%) reported gastrointestinal complaints, 32 (80%) reported upper respiratory complaints, and 33 (82.5%) reported lower respiratory complaints. All (100%) of the patients reported complaints in multiple-organ systems. 39 (97.5%) reported either upper or lower respiratory complaints. When asked to assess their overall health compared to other people of similar age, 16 (40%) rated their overall health as poor, 16 (40%) rated their health as fair, and 4 (10%) rated their health as good, 1 (2.5%) rated health as excellent, and 1 person did not respond.

Telephone calls were made to all 40 patients; 25 patients were successfully contacted and voluntarily agreed to participate in the study. 2 patients did not respond

because they were critically ill with unrelated conditions, 13 patients could not be reached at the address or telephone number provided. The baseline characteristics of the respondents are included in Table 1. The mean age of the respondents at the initial visit was older (42.1) than the group as a whole (34.9). The patients in the responding group versus the non-responders reported similar numbers and types of medical complaints.

Table 1. Baseline characteristics of the 25 patients who completed the study

Age	42.1 +/- 12
Female	72%
Asthmatics	24%
Respiratory Sx	96%
No-specific Sx	100%
Exposure at work	67%
Exposure at home	33%

At the time of interview, 12 people moved (or left the mold exposed work environment), and 7 people indicated that their environment was remediated to their satisfaction and they were in the same work or home location. Thus, a total of 19 persons are considered in the “remediated” group. The level of improvement in symptoms was similar in both subgroups, although slightly more improvement was reported in the group that indicated that they were able to successfully remediate and could stay in their home or job (figure 1).

Figure 1. Relative risks of no improvement in symptom categories for non-intervention vs intervention (moved or remediated)

	RR, (95% CI)
Total Symptoms Change	4.75 (1.99 - 11.35)
Non-specific Symptoms Change	3.80 (1.79 - 8.06)
Respiratory System Change	2.38 (1.40 - 4.02)
Overall health Change	3.96 (1.54 - 10.15)

Of the 19 who reported that they were no longer exposed to mold, 4 subjects experienced worsening or unchanged overall health, while the remaining 15 experienced improvement in overall health. 15 experienced improvement in total symptoms, 14 experienced improvement in non-specific symptoms, and 11 experienced improvement in respiratory symptoms. Thus, improvement occurred in the respiratory, non-specific symptoms, and in all symptoms combined. There was no significant interaction between gender and remediation status using a logistic regression analysis.

One person indicated a partial remediation that was unsuccessful and 5 people indicated that there was no change in their environment, placing 6 subjects in the “unremediated” group. All 6 experienced worsening of some symptoms, 5 experienced worsening of non-

specific symptoms, 5 experienced worsening respiratory symptoms, 4 indicated worsening of overall health, 1 had no change in overall health and 1 indicated improvement in overall health.

Six patients were being treated by their physicians for asthma at their initial clinic visit. All either moved or remediated their mold exposure. All experienced some improvement in either overall health assessment or medical symptoms, 4 experienced improvement in all respiratory and non-specific symptoms score and overall health assessment (figure 2).

Figure 2. Relative risks of no improvement in symptom categories for moved vs. remediated (within the intervention group)

	RR, (95% CI)
Total Symptoms Change	1.75 (0.22 – 13.76)
Non-specific Symptoms Change	2.33 (0.32 – 16.95)
Respiratory System Change	0.97 (0.33 – 2.88)
Overall health Change	1.75 (0.22 – 13.76)

DISCUSSION/CONCLUSION

The results described above indicate a significant improvement in broad categories of symptoms and self reported global health assessment for those subjects who moved away from or remediated mold conditions. This effect occurred in the res-

piratory or allergy-mediated symptoms as well as in the non-specific symptoms. We would expect to see an improvement in those people with clearly documented mold allergies upon cessation of exposure. The relationship between mold exposure and non-specific symptoms (abdominal pain, headache, etc.) is more controversial. This trend is interesting because documentation of relationships between intervention and symptom outcome has been sparse, and often refers to individuals with complaints that persist long after the exposure has resolved. While our data is limited to a small clinical cohort, it clearly suggests that non-specific symptoms resolve concomitantly with, and occasionally despite lack of resolution of pathophysiologically explained respiratory symptoms. This is consistent with the hypothesized acute cause and effect relationship between mold exposure and non-specific symptoms.

The observed associations in our study were subject to several biases, including exposure misclassification, selection biases, biases in recalling and reporting past and present symptoms, and regression towards the mean. Actual exposure to mold was not confirmed by personal monitoring to assess the degree of mold inhalation. Remediation of the mold condition was not objectively confirmed and it is possible that mold exposures may have decreased without any specific intervention. Since intervention was not randomized, subjects who did not have an intervention may have been more likely to have home or work conditions that did not represent significant mold exposures. Their symptoms may have been due to some other condition(s), including chronic conditions that would be unlikely to improve over time. On the other hand, if the misclassification were non-differential, the bias would be towards the null hypothesis.

Differential recall and symptom reporting biases might have biased the results towards positive findings. Subjects, who remediated or moved away from exposure, perhaps at great personal expense or inconvenience, may be more likely to report improvement in their symptoms. Intervention was recommended at most initial visits when the examining physician felt that the symptoms were related to mold exposure, so subjects may have been biased in reporting improvement when contacted by a clinic representative or the examining physician at a follow-up interview. Regression towards the mean can lead to spurious improvement in symptoms in studies that use data from a clinical visit as a baseline for comparison with later collection of symptom data. However, this bias is more likely to be non-differential, biasing the results towards the null hypothesis.

Since the subjects were not blinded, intervention may have had a significant placebo effect, which may be especially important in a study of symptomatic outcomes. Furthermore, the non-specific intervention of moving, experienced by 12 of the 19 subjects in the intervention group, may have reduced other confounding expo-

tures that may have been responsible for the symptoms attributed to mold in the home or workplace.

Despite the limitations of this study, the results are consistent with other studies and reports showing improvement in symptoms with decreased mold exposure, although these results of these studies may be subject to many of the same biases. Several studies demonstrate a relationship between mold and asthma and allergy in adults and children with improvement after cessation or decreased exposure to mold (Jaakkola, N. *et al.* 2002; Jaakkola, J. J. F., Jaakkola, N., 1993; Seuri *et al.* 2000; Dales *et al.* 1991). Two recent reports suggest improvement in other symptoms after cessation of mold exposure. Johanning *et al.* reported that primary disorders of the respiratory system, skin, mucous membranes, and central nervous system occurred in a cohort of 147 ambulatory adults and children with indoor mold exposure. In the sentinel case evaluation of one mold-contaminated apartment, all subjects reported a marked improvement in medical symptoms and a partial resolution of their chronic health problems. In addition to improvement in respiratory symptoms, improvement in headaches, fatigue, and flu-like symptoms was reported in the whole cohort, however no statistics were performed (Johanning *et al.* 1999). Ebbehov *et al.* performed an intervention study where a public indoor swimming pool with a leaky roof was renovated and the mold was remediated. 25 subjects were asked about 4 irritative symptoms (irritated eyes, nose, throat, and eczema), and five general symptoms (headache, fatigue, lack of concentration, dizziness, and feeling heavy in the head). The investigators noted a statistically significant fall in the number of symptoms that appeared to coincide with the mold removal. However, there was no control group in this study (Ebbehov *et al.* 2002).

While the mechanism of symptom improvement is not clear, we found a statistically significant relationship between interventions to decrease mold exposure and improvement in overall health assessment, respiratory, and non-specific symptoms. It is possible that this association may be due to recall bias, reporting bias, exposure misclassification, a placebo effect, or a Hawthorne effect. However, this work supports the conclusion that mitigating mold exposure is appropriate advice to provide to a patient who is concerned about symptoms from mold exposure. Further controlled studies and exposure measurements are needed to confirm the effect that we report, as well as begin to evaluate potential mechanisms for health effects of mold exposure that are not caused by allergy.

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Chapter 4

Health Effects IV – Experimental Research

Session chairs:

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CYTOTOXICITY AND INFLAMMATORY RESPONSES INDUCED BY MOLDY HOUSE MICROBES

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The indoor air microbial concentrations in moldy buildings are only slightly elevated when compared to those in reference buildings, but the detected microbial levels do not necessarily correlate with the reported adverse health effects. Thus, the causal relationship between detected exposures and health end points are not known. Based on current epidemiological data, it can be assumed that one of the main contributing factors in the etiological mechanism of the reported symptoms is inflammatory response towards specific organic materials in the microbes. This would include activation of immunological and epithelial cells leading to increased production of cytokines, NO and reactive oxygen species (ROS) and subsequent cytotoxicity. These mediators play an important role in regulating pathophysiology of inflammatory diseases including asthma. Moreover, microbes growing on moist building materials are well known to produce toxins. At present there is serious lack of data based on biochemical evidence of a link between objective biomarkers, qualitative characteristics of the microbial emissions and subjective symptoms. These data are, however, needed for proper risk assessment of the moldy house problem, the measures taken to solve it and assessment of its importance for public health and health economy.

The overall aim of our research is to find out which microbes among the mixed population of the microbes present in the moldy houses are able to cause adverse respiratory health effects and what are the mechanisms of them. The focus is on inflammatory responses and cytotoxic effects 1) in human and mouse cells, 2) in a mouse model and 3) in upper and lower airways of people living or working in moldy buildings. Based on our recent findings *in vitro*, bacterial strains especially mycobacteria and streptomycetes are significantly more potent inducers of inflammatory mediators, such as proinflammatory cytokines and nitric oxide, both in human and mice cells than the fungal strains. Some of the fungal strains (*S. chartharum*, *A. versicolor*) and streptomycetes can cause cytotoxicity in these cells, whereas mycobacteria decrease only moderately the cell viability. Moreover, the growth and sporulation environment of microbes, including pH and nutrients in

culture media or growth conditions provided by building materials, strongly affect on the ability of the microbial spores to induce inflammatory responses and cytotoxicity in mammalian cells. Thus, the growth condition needs to be carefully considered when evaluating the inflammatory potential and/or toxicity of these organisms. In line with these results, we have demonstrated that, the same microbes, which induce production of inflammatory mediators and/or cytotoxicity in mice cells *in vitro*, cause inflammation and cytotoxicity in the lungs of mice after an intratracheal exposure to single dose of spores of these microbes. These microbes, however, differ significantly from each other in potency, time-course, and induced spectrum of inflammatory mediators. The production of the same inflammatory mediators (i.e. NO, cytokines) were detected also in nasal lavage fluid (NAL) in association with respiratory symptoms and prolonged working period of subject in a mold damaged school building.

DO MVOCS CAUSE IRRITATION?: NASAL EFFECTS OF VOCS AND VOC OXIDATION PRODUCTS IN CONTROLLED HUMAN EXPOSURES

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ABSTRACT

Concentrations of microbial volatile organic compounds (MVOCs) in indoor air are low compared to levels of volatile organic compounds (VOCs) known to cause acute irritation in animals and humans. Some MVOCs may react with low levels of ozone to create potentially irritating compounds. We hypothesized that a mixture of 23 VOCs would cause more nasal irritation and inflammation than a clean air (CA) control, and that addition of ozone (VOC+ozone) would enhance these effects. In this study, 130 healthy women completed three 135-minute exposures. VOC and ozone concentrations were 25 mg/m³ and 40 ppb, respectively. The CA exposure was masked with an initial pulse of VOC mixture (2.5 mg/m³). We found no significant differences in nasal irritation symptoms or nasal lavage PMNs between VOC+ozone, VOC and CA conditions. Effective blinding to the exposure conditions may explain the negative symptom findings. MVOCs appear to be unlikely to cause acute upper respiratory irritation in mold-contaminated buildings.

INDEX TERMS: Microbial volatile organic compounds, Controlled exposure, Irritation, Inflammation

INTRODUCTION

Microbial volatile organic compounds (MVOCs) and their oxidation products have been implicated as a potential cause of the eye, nose, and throat irritation reported by occupants of buildings with mold growth or dampness (Husman, 1996). A wide variety of MVOCs are produced by fungal species found indoors, including alkanes, alkenes, ketones, organic acids, and terpenes (Wilkins, Larsen, Simkus, 2000). Concentrations of MVOCs measured in the air in mold-contaminated buildings are typically in the $\mu\text{g}/\text{m}^3$ range (Pasanen, Korpi, Kasanen *et al.* 1998). It is difficult to estimate the contribution of MVOCs to total volatile organic compounds (VOCs) in indoor air, because most MVOCs are not unique to mold.

Although no epidemiological studies have directly linked measured exposure to MVOCs with irritation symptoms, studies of controlled animal and human exposures indicate that VOCs can cause irritation. Inhalation of relatively high levels of VOCs can cause upper respiratory tract irritation in animals, as measured by inhibition of respiration in the "Alarie" mouse bioassay (Schaper, 1993). Using this assay, 5 MVOCs were found to have relatively low irritation potency, but the mixture had a 3.6 fold greater potency than that predicted by its components, suggesting possible synergy in mixed exposures to VOCs (Korpi *et al.* 1999). Controlled human exposure studies have demonstrated sensory irritation from unblinded exposure to a mixture of 22 VOCs at $25 \text{ mg}/\text{m}^3$ ($\sim 7 \text{ ppm}$), at the high end of what may be found in poor-quality indoor air (Hudnell *et al.* 1992; Prah 1998). Using PMNs in nasal lavage (NL) fluid as an objective marker of inflammation, other studies using similar exposures have yielded contradictory results (Koren, Graham, Devlin, 1992; Pappas *et al.* 2000). In one study, 14 subjects had a 2-fold increase in NL PMNs immediately following a 4-hour exposure at $25 \text{ mg}/\text{m}^3$ (Koren, Graham, Devlin 1992), whereas in another study there was no significant increase in PMNs in 15 subjects after similar exposures at $25 \text{ mg}/\text{m}^3$ and $50 \text{ mg}/\text{m}^3$ (Pappas *et al.* 2000).

The irritation potency of some VOCs may be enhanced by oxidation reactions that occur in indoor air (Wainman *et al.* 2000; Weschler, 2000). Some unsaturated MVOCs, such as terpenes and 1-octene-3-ol, may be rapidly oxidized in indoor air. Low-level ozone may react with terpenes in indoor air to form a complex mixture of potentially irritating products that include hydroxyl radicals, aldehydes, carboxylic acids, and fine particles (Wainman *et al.* 2000; Wolkoff *et al.* 2000). Animal inhalation studies have demonstrated that these reaction products are considerably more irritating than their precursors (Wilkins *et al.* 2001), but there have been no prior controlled studies of human exposure to VOC-ozone reaction products.

This study was designed to evaluate symptomatic, inflammatory, and psychophysiological effects of controlled human exposure to a mixture of 23 VOCs, with and

without ozone at 40 ppb. The VOC mixture included several compounds that have been identified as MVOCs including d-limonene, alpha-pinene, 2-propanol, and 1-butanol (Pasanen *et al.* 1998; Wessen, Schoeps, 1996). Several other components are likely MVOCs, although they have not been specifically reported. This report will focus on a preliminary analysis of nasal outcomes, including nasal irritation and PMNs in NL fluid.

METHODS

The subjects were 130 normal, healthy females with mean age 27 years. They were nonsmokers with no history of asthma or other pulmonary disease, chemical sensitivity, or other serious disease. Subjects were evaluated for allergy status by a questionnaire and RAST test for 5 locally important aeroallergens. Subjects were scored on measures of self-reported chemical odor intolerance and negative affectivity. Informed consent was obtained for each subject prior to participation, and the protocol was approved by the UMDNJ-Robert Wood Johnson Medical School Institutional Review Board.

All subjects had one exposure to each of the three 135-minute exposure conditions (“clean” air (CA), VOC, and VOC+ozone) in random order, with at least one week between exposures. Half of the subjects were randomly assigned to “stressor” task (public speaking) at 60 minutes from the start of exposure. Exposures took place in the EOHSI Controlled Environment Facility (CEF), a 25 m³ stainless steel room. The composition and relative weight of the VOC mixture used has been previously reported (Fan *et al.* 2003). Twenty-two of the 23 compounds are the same as those used in human exposure studies reviewed above. The additional compound was d-limonene, the most frequently identified terpene in indoor air. The VOC mixture was flash evaporated and injected into the air supply. The total VOC concentration in the air of the indoor environment, approximately 25 mg/m³, was achieved by adjusting the flow rate of the delivery air. Ozone generated by an ozone generator was delivered into the CEF to maintain a concentration of 40 ppb. The CA condition was masked by a low-concentration (~ 2.5 mg/m³) pulse of VOCs during the first five minutes of exposure. Due to low air exchange rates we had difficulty controlling temperature and relative humidity, which ranged from 72°F to 80°F and from 20% to 48% respectively.

Thirty-seven symptoms including “nasal irritation, dryness, or itching” were rated before, after, and at 4 time points during exposure, using a ratio scale ranging from 0 (“no sensation”) to 100 (“strongest imaginable”)(Green *et al.* 1996). Nasal lavage with normal saline was performed before and after exposures using a nasal spray technique (Noah *et al.* 1995). Briefly, a nasal metered dose inhaler (100 µL per spray) was used to wash out one nostril while the other was held occluded. After 5

sprays, the subject gently exhaled through the washed nostril, expelling the lavage fluid into a cup. This was repeated 8 times in each nostril for a total of 8 ml of saline, of which approximately 50% was recovered. The sample was immediately placed on ice and processed within 2 hours. Cells were counted on a Coulter Counter. Differential counts of cytocentrifuged (800 x g) cells were performed using a modified Wright's stain (Dif-Quik) by a single technician who was blind to exposure status.

For this preliminary analysis of the nasal irritation symptom data, we analyzed only the no-stress group (n=61) to avoid any interaction with the psychological stressor. We dichotomized the nasal irritation symptom response into whether the symptom was reported or not. We assume that the trend in the odds of symptom reporting over time should follow a predictable smooth path. This assumption is worked into the model by including time as a continuous covariate. A hierarchical logistic regression modeled the probability of no symptoms being reported for assessment at baseline to after removal from exposure chamber. In this model, the symptom report at 20 minutes prior to chemical exposure was assumed to be representative of symptoms just prior to the onset of the chemical exposure (time zero).

For NL cell counts, repeated measures ANOVAs were used to analyze the effect of exposure, with a random effect to account the correlation between measurements within the same individual. The variable used for the response in the analysis is the difference of the log-transformed values of the counts/percentages after exposure and at baseline. If a variable was recorded as zero, 0.1 was added to it to make the log-transform possible.

RESULTS

Figure 1 summarizes the mean “nasal irritation, dryness, or itching” symptom score at collection time-points before, during and after the 3 exposure conditions for 61 subjects who did not receive the psychological stress condition. Although the mean symptom reports appear slightly elevated in the VOC and VOC+ozone conditions, compared to the “clean air” condition, this was not statistically significant. As measured on the 0 to 100 point scale, the magnitude of mean symptom increase for all conditions was very modest.

Figure 1. “Nasal irritation, dryness, or itching” symptom time course for each exposure condition

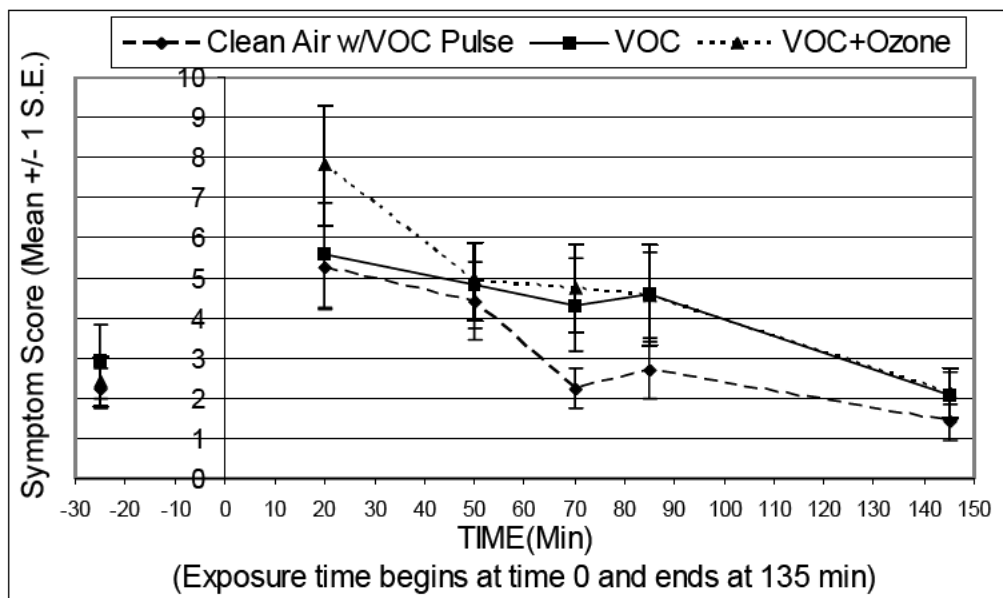


Table 1 provides the means and confidence intervals for the relative changes in the concentration of PMNs in NL fluid for each exposure, based on the least square means of the log-transformed values, calculated within the framework of the repeated measures ANOVA. The means and the endpoints of the confidence intervals are transformed into the relative changes ($\% = (\text{post-pre})/\text{pre}$). There was a relative decrease in PMNs for all conditions, probably due to the “wash-out” effect of the initial lavage. There are no significant differences between exposures.

Table 1. PMN concentrations in nasal lavage fluid (cells/ml)

Exposure	Mean Relative Change	95% CI	Exposure Effect p-value	p-value for comparison with clean air
CA	-77.8%	(-91.6%, -41.3%)	0.909	--
VOC	-83.7%	(-93.8%, -56.8%)		0.663
VOCO	-80.9%	(-92.8%, -49.6%)		0.828

DISCUSSION

Our preliminary results indicate that exposure to the VOC condition did not cause a significant increase in nasal irritation symptoms (Figure 1). Other studies have reported a significant increase in mucous membrane symptoms with exposure to very similar mixtures of VOCs at the same concentration (Prah, 1998; Hudnell *et al.* 1992) or only at twice the concentration (Pappas *et al.* 2000). Whereas in earlier studies no attempt was made to blind subjects to exposure to the odorous mixture, the initial pulse of VOCs during our CA exposure effectively blinded the subjects to exposure, confirmed by subjects' inability to correctly guess the exposure condition. We found a similar modest increase in nasal symptoms for all conditions, suggesting that increased reporting of subjective symptoms may have been mediated by olfaction rather than by trigeminal irritation.

VOC and VOC+ozone exposures did not cause an increase in PMNs in NL fluid (Table 1). This result contrasts with a study of 14 healthy volunteers that found a significant two-fold increase in PMNs in NL immediately after a 4-hour exposure to a mixture of 22 VOCs at 25 mg/m³ (Koren, Graham, Devlin, 1992). However, it is consistent with another study that found no significant increase in PMNs in 15 subjects at 2 hours after 4 hr exposures to the same VOC mixture at 25 mg/m³, as well as at a concentration of 50 mg/m³ (Pappas *et al.* 2000). Differences in time-course and technique may explain some of the differences in results. Our exposure period was shorter, with post-exposure NL taking place about 3 hours after the start of the 135-minute exposure. However, it seems unlikely that this would fully explain the lack of positive findings in a study that had greater statistical power than the previously reported positive study. The earlier studies used a "bolus lavage" method, in contrast to the nasal spray method used in this study. The effect of different NL technique cannot be evaluated in the present absence of controlled studies comparing the two techniques.

Chemical analysis confirmed the formation of potentially irritating compounds such as aldehydes and fine particles in the VOC+ozone exposure as reported earlier (Fan *et al.* 2003), yet we found no significant differences in nasal irritation or PMNs between the VOC+ozone, VOC and CA exposures. Perhaps this result is not entirely unexpected, given that the concentrations of ozone (40 ppb) and terpenes (300 ppb) were several orders of magnitude lower than concentrations used in the animal irritation studies (3 ppm and 50-ppm, respectively) (Wolkoff *et al.* 2000). However, our VOC concentrations were at the upper end of the range of total VOC concentrations that might be found in poor quality indoor air, and well above levels of MVOCs reported in indoor air (Pasanen *et al.* 1998). Our results are consistent with models showing that application of the animal study results to

reported indoor MVOC concentrations resulted in a very low probability of human irritation (Pasanen *et al.* 1998).

CONCLUSION AND IMPLICATIONS

Brief, one-time exposures to a mixture of low-level VOCs and their oxidation products do not appear to cause significant acute increases in nasal irritation or inflammation in healthy subjects, as measured by symptom report and PMNs in NL fluid. The VOC mixture contained several known MVOCs. Although the irritation potency of complex and variable mixtures of VOCs, MVOCs, and their oxidation products is difficult to predict, reported air concentrations of MVOCs in mold-contaminated buildings are much lower than the VOC concentrations used in this study. Therefore, our results support the conclusion that MVOCs are unlikely to be a significant cause of acute upper respiratory irritation in mold-contaminated buildings. However, the results of this study may have limited applicability to longer-term, repeated exposures to VOCs and MVOCs that occur in homes and workplaces.

ACKNOWLEDGEMENTS

This study was funded by NIOSH 5R010H03691 and NIEHS P30ES05022

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OCULAR EFFECTS IN HUMANS BY EXPERIMENTAL EXPOSURES TO DIFFERENT TYPES OF DUST

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ABSTRACT

Healthy subjects (non-allergic with nasal histamine hypersensitivity, non-allergic with normal sensitivity, and pollen-allergic with or without nasal hypersensitivity) were exposed for 3 hours in a climate chamber to clean air ($< 10 \mu\text{g}/\text{m}^3$ total suspended particle, TSP), house dust at $500 \mu\text{g}/\text{m}^3$ TSP, dust $500 \mu\text{g}/\text{m}^3$ TSP with added β -1.3-glucan (intended $50 \mu\text{g}/\text{m}^3$), and dust $500 \mu\text{g}/\text{m}^3$ TSP with added aldehydes corresponding to $300 \mu\text{g}/\text{m}^3$ aldehydes in the air. No significant effects of the exposures or subject groups were found in Break Up Time (BUT), epithelium damage score, and Trolox Equivalent Antioxidant Capacity in tear fluid (TEAC). The subgroup analysis showed a decrease of BUT the day after exposure to dust containing glucan, and TEAC increased subacutely and decreased the next day after exposure to dust containing aldehydes. Significant correlations were found between several subjective responses. These results may indicate that aldehydes or glucan change the toxic potential of airborne dust but may also be chance findings but suggest that the hypotheses should be tested in further studies

INDEX TERMS: Ocular effects, humans, dust, glucan, aldehydes.

INTRODUCTION

The background for the study is the frequent complaints of poor indoor air quality and the associations found in epidemiological studies between complaints of house dust in the air and water damage and/or microbial growth indoors. The paper is focused on objective physiological changes in eyes caused by exposure to

house dust, dust added with glucan, and dust with added aldehydes. Tests of the association between the objective symptoms and the subjective responses were included in the paper. The results are part of a larger study with more health effects measures. The results of that study is under publication elsewhere. Other results from the same study are found in Mølhave *et al.* (2003, 2004). The study is a continuation of a previous study (Mølhave *et al.* 2000 a, b, 2002 a, b).

The main hypotheses for this paper were: (1) dust exposure causes physiological effects on human eyes, (2) adsorption of VOCs (exemplified by aldehydes) on dust increases the toxic potential of the dust, (3) contamination of dust with β -(1.3)-D-Glucan increases the toxic potential of the dust, and (4) the subjects respond differently to these exposures depending on personal factors.

MATERIALS AND METHODS

Subjects (36) were selected from about 150 volunteers to one of three subgroups (non-allergic with nasal histamine hypersensitivity, non-allergic with normal-sensitivity, and pollen-allergic with or without nasal hypersensitivity). Each group included 12 subjects. The histamine sensitivity was defined as an increase in nasal mucosal swelling of at least 0.6 mm after 2 and 5 min after nasal installation of histamine. The nasal histamine provocation test was performed and measured as previous reported (Juto, 1985).

The main hypothesis of eye effects caused by dust exposures was tested in experimental exposures of the subjects in a climate chamber. The study included three replications of the same basic design. For each replication, 12 subjects were assigned to four exposure order sub-groups. The four subgroups of three persons include one person from each of the three groups mentioned above. Each subgroup was exposed to four types of exposures in a balanced 4x4 Latin-square design. In all 36 subjects were examined.

The dust used for the exposures was sampled in office buildings or institutions without known indoor climate problems (Mølhave *et al.* 2000a). The dust was collected in a period without pollen production according to the local allergen monitoring station. The collection and preparation followed a standardized procedure (Mølhave *et al.* 2000a). Bulk dust was spiked with a mix of three aldehydes: n-hexanal, n-nonanal, and n-decanal corresponding to 300 $\mu\text{g}/\text{m}^3$ Total Volatile Organic Compounds (TVOC) at 500 $\mu\text{g}/\text{m}^3$ dust in the air or with β -(1.3)-glucan from WAKO Incorporated (Germany). To 100 g of bulk dust was added 1g of glucan.

Exposures were (1) Clean air (Dust < 10 $\mu\text{g}/\text{m}^3$ total suspended particle, TSP), (2) House dust at 500 $\mu\text{g}/\text{m}^3$ TSP, (3) House dust 500 $\mu\text{g}/\text{m}^3$ TSP with added glucan

(intended 50 $\mu\text{g}/\text{m}^3$), and (4) House dust 500 $\mu\text{g}/\text{m}^3$ TSP with added aldehydes corresponding to 300 $\mu\text{g}/\text{m}^3$ in the air. The duration was four hours.

The exposure was performed in a 32 cubic meter stainless climate chamber (Mølhave *et al.* 2000a) using a dust generator, which was placed outside the chamber and was slightly modified from a previously developed acoustic generator (Mølhave *et al.* 2000a).

The exposures were arranged in daily 6-hour sessions, covering one hour clean air for acclimatization, four hours in the chamber and one hour post exposure sampling. The four hours climate chamber exposure included clean air (30 min), increasing exposure (30 min) and three hours of exposure at an equilibrium concentration.

This study was performed according to the Helsinki declarations (Mølhave, 1998) and was accepted by the local committee for ethical use of human subjects in experimentation (Reference number 1999/4486).

Effect measurements: This paper reports the ocular measurements, which included tear film stability (BUT), epithelium damage score (ED), and Trolox Equivalent Antioxidant Capacity (TEAC) in tear fluid. In addition, some of the subjective perceptions or symptoms ratings related to the eyes and investigated by questionnaire are also included in this paper other results from the same study are found in Mølhave *et al.* (2003, 2004).

Tear film stability or Break-up time (BUT) was performed using a slit lamp (model TopCon SL 75) and was measured as time (sec) from an eye blink to the appearance of visible break-up of the tear film after instillation of 10 μl Na-flouroscein (Norn, 1983).

Conjunctival Epithelial Damage (ED) was measured as the number of dots in the intact epithelium using a slit lamp after staining with 10 μl , 1% Lissamin green in the lower conjunctival sack (Norn, 1983). After a blinking period, the number of dots was counted and categorized in following four groups: A. 0-10; B. 11-50; C. 51-100; D>100 for the lateral, medial and inferior conjunctival and corneal (Franck, 1986, Kjærgaard *et al.* 1989).

Trolox Equivalent Antioxidant Capacity (TEAC) in tear fluid was measured on tear samples. 10 μl tear fluid was sampled from the outer eye canthus using a calibrated capillary tube. The enhanced chemiluminescent reaction method (Whitehead *et al.* 1992, Kjærgaard *et al.* 2004) was used for the TEAC measurement.

Tear film stability or Break-up time (BUT) and Conjunctiva Epithelial Damage (ED) were measured together once the day after the exposures to avoid the impact

of colour staining on other measurements such as nasal lavage measurements. Tear fluid TEAC measurements was made before exposure, after exposure (subacute), and the next day (delayed) in each exposure session.

Subjective evaluations were carried out by a computer-based questionnaire, included 29 questions. The subjects scored their evaluation on a PC- screen by placing a marker on a 130 mm long visual analogue scale. The intensity of any discomfort was registered as the length in mm from the left end of the scale to the marker. (Mølhave *et al.* 1986). For this eye related part of the study the following questions were extracted; illumination, glare to the reflex, air quality, need of ventilation, eye irritation, dry eyes, runny eyes, headache, concentration difficulty, general well-being, and stressed induced by chamber occupancy. The questionnaire ratings were measured four times, i.e. before the exposure as a baseline and additional three times during the exposure; just at the start, in the middle, and just before the termination of exposure.

Statistics: Since these effects were not primary hypotheses of the statistical analysis should be considered exploratory, and thus useful only for generating hypothesis for examination in further studies. To reduce the between-subject variation, TEAC values and questionnaire data were baseline corrected by subtraction of the value of the variable before entrance into the climate chamber. For the BUT and ED no baseline value was obtained, hence correction was not possible. The four main hypotheses mentioned above were tested for each effect variable as an independent analysis. The analyses included a preliminary analysis of data quality, distributions and an analysis of the effects. Following transformations when required a more detailed (e.g. multivariate) analysis was made. The repeated type GLM analysis was used to test for differences between different exposures and subject groups. Age, gender and exposure blocks were included as co-factors Data distribution analyses, GLM analyses, and correlation analyses were made using SPSS 10.0. A p-value of 5% was considered statistical significant.

RESULTS

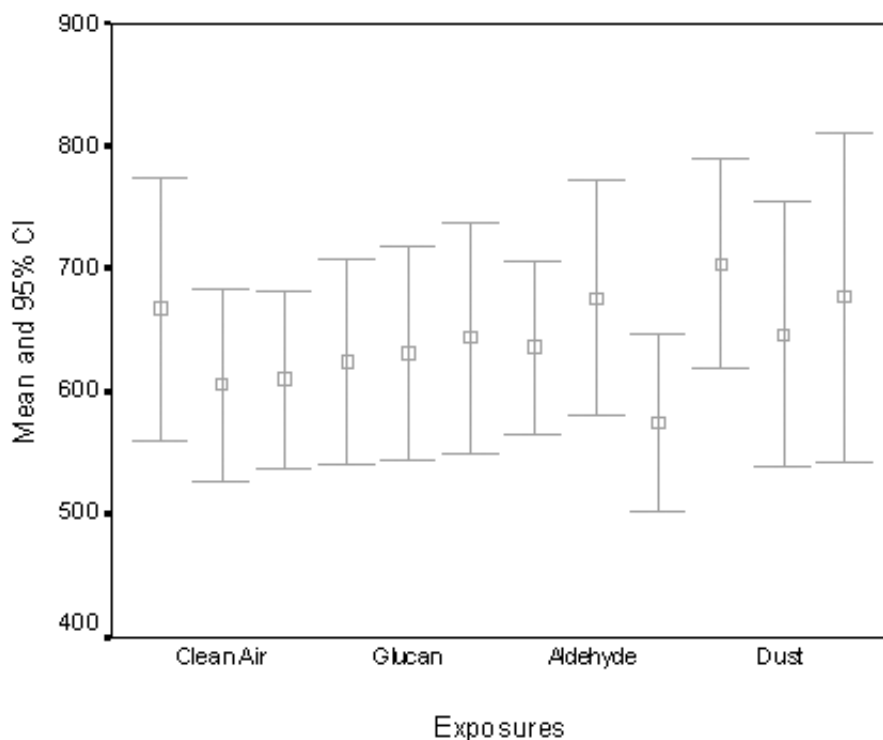
The data was obtained from all 36 subjects, four exposure levels and three blocks.

No significance effects of co-factors were found, no significance was found in break-up time and epithelium damage score between the four kinds of exposure or three groups of subjects. Only in the analysis comparing each exposure with clean air, BUT decreased significantly during exposure to dust containing glucan ($p=0.039$). No significance was found related to ED.

For tear TEAC (Fig 1), similarly, no significance was found between exposures or subject groups for the three sessions of measurements. However, the average

TEAC (Fig. 1) at baseline, subacute, and delayed measurements indicate that TEAC increased subacutely ($p=0.012$) and decreased at the next day significantly for dust with added aldehydes.

Figure 1. Average TEAC values (mean and 95%CI) for 36 subjects during the exposures. (The cluster represents before, just end, and the day after exposure, respectively).

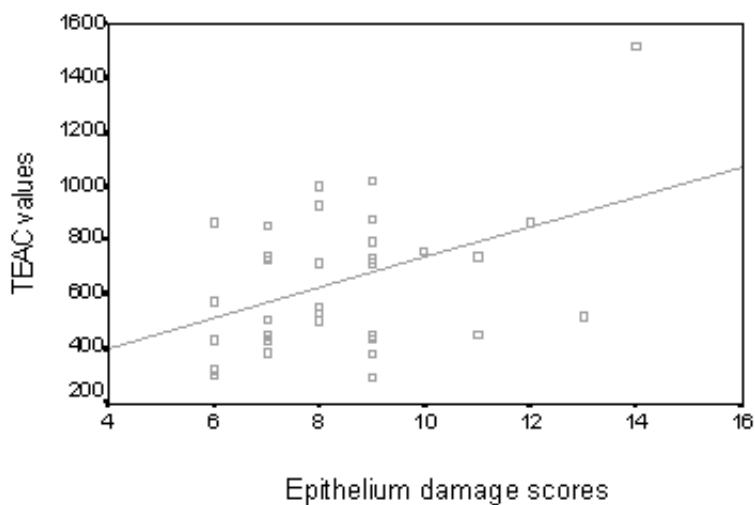


Correlation analyses were made between the objective physiological measurements, between relevant subjective perceptions and symptoms and between objective vs. subjective measurements. Data for BUT, ED, and tear TEAC measurements at the day after the exposures were used for correlation analysis of objective measurements. Data of baseline corrected ratings of questions at the end of the exposure were used for the subjective measurements. Baseline corrected ratings of questions and tear TEAC measures at the end of the exposure together with BUT and ED measurements at the day after the exposure were used for the analyses between objective and subjective measurements.

No correlation was found between BUT and the two other objective measurements ED and TEAC whereas TEAC was significantly correlated with ED during

the glucan exposure ($r=0.407$, $p=0.014$, Fig. 2). This correlation disappears if one outlier is excluded from the analysis.

Figure 2: The correlation between tear TEAC and ED measurement during the exposure to dust containing glucan.



The GLM analyses for questionnaire data showed that perceived eye irritation significantly worsened during exposure ($p=0.016$). These self-reported perceptions were included in the analyses of the association with objective measurements. The subjective responses used in the correlation analysis were the data registered just before the end of exposure because these ratings were closest in time to the BUT and ED measurements. The perception ratings were highly interrelated, whereas few correlations were found between the subjective responses and objective measurements.

DISCUSSION AND CONCLUSION

The objective measures BUT and ED were only measured once at the day after exposure, because the vital staining could effect the other measurements, especially the lavage. This limited the value of the analysis reported in this paper. The analysis show no major differences between the subject groups or between clean air and the different dust exposures. The overall hypotheses about different effects between different groups and dust exposures could not be supported. The results from the subgroup analyses provide clues for further studies, i.e. if glucan and aldehydes may modulate the health effects of humans by indoor exposures to house dust. These indications are supported by other findings from the same study which show nasal changes caused by mild inflammation (publications in progress or Mølhave *et al.* 2003, 2004a, b).

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COMPARISON OF THE ACUTE EFFECTS OF *S. CHARTARUM* AND OTHER INDOOR MOLDS ON THE LUNGS OF INFANT RATS

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ABSTRACT

Stachybotrys chartarum has been linked to adverse health effects such as sick building syndrome and cases of pulmonary hemorrhage in infants in Cleveland. This fungus is capable of producing potent mycotoxins including trichothecenes. While the original case-control study of the Cleveland infants found the highest odds ratio for airborne *Stachybotrys chartarum*, grouping of "other fungi" was also significant (Etzel *et al.* 1998). The goal of this study was to compare the *in vivo* effects of *S. chartarum* with those of other environmental fungi.

Two isolates of *S. chartarum* JS 58-06 (no satratoxin) and JS 58-17 (high satratoxin), *Penicillium chrysogenum*, *Penicillium verrucosum*, *Paecilomyces variotti* and *Paecilomyces lilacinus*, and *Aspergillus ochraceus* were studied. All fungi were grown at room temperature on potato dextrose agar for 3 weeks. Spores were characterized with respect to their cytotoxicity, proteolytic activity and viability. Seven d old rats were exposed via tracheostomy to 2×10^5 spores/gm BW of different fungi (8 pups/group). Control animals (n=8) received phosphate buffered saline (PBS). The animals were sacrificed 48 h later and bronchoalveolar lavage (BAL) was performed along with lung histology. The levels of leukocytes, erythrocytes, proinflammatory cytokines and protein in BAL fluid were quantified.

The spores of *S. chartarum* were the most cytotoxic and had the highest proteolytic activity and viability. All the animals exposed to the viable spores of highly toxic strain JS58-17 died before 48h. The same dose of the hemolytic strain JS 58-06 did not lead to death but caused effects that were much more severe than those of other fungi. Lung inflammation observed histologically in the animals treated with other fungi was much more mild com-

pared to those treated with *S. chartarum*. Inflammatory indices were significantly elevated ($P < 0.05$) in the BAL fluid from the animals treated with most of the fungi, the highest being for *S. chartarum*. However, *A. ochraceus* was an exception in that many of the inflammatory indices were at or below the levels observed for PBS treated controls, suggesting an inhibitory effect. BAL fluid protein, neutrophils and cytokine concentrations were the most sensitive indicators of exposure.

INDEX TERMS: *Stachybotrys chartarum*, indoor mold, infant rats, pulmonary effects.

INTRODUCTION

Stachybotrys chartarum became the center of attention after an association was found between the presence of this mold and cases of pulmonary hemorrhage in infants living in water damaged houses in Cleveland, Ohio (Etzet *et al.* 1998). The main concern about *Stachybotrys chartarum* stems from trichothecene toxins produced by some strains. These are the most potent known protein synthesis inhibitors (Miller *et al.* 2003). Exposures to *S. chartarum* toxins are known to have serious health effects in animals. Severe, even fatal cases of stachybotrytoxoses caused by ingestion and particulate inhalation from contaminated feed involving leukopenia and toxic aleukia have been described. Because of its very high water requirement, *S. chartarum* is often regarded as an indicator fungus, whose presence in a particular environment connotes significant water intrusion. While the original case-control study of the Cleveland infants found the highest odds ratio for airborne *S. chartarum* (9.8, CI 1.1-3x10⁶), grouping of "other fungi" was also significant (OR=1.06, CI 1.00-1.16). The goal of this study was to compare the *in vivo* effects of *S. chartarum* with those of other environmental fungi. In addition, two strains of *S. chartarum* with different trichothecene toxicity were selected to evaluate the role of trichothecenes in pulmonary pathophysiology.

METHODS

In addition to two Cleveland isolates of *S. chartarum*, JS 58-17 (high satratoxin producer from a control infant home) and JS 58-06 (no satratoxin, high hemolysin producer from the home of an infant with pulmonary hemorrhage), five other common indoor molds were used in this study. Two isolates of *Penicillium chrysogenum* came from the lungs of patients with pulmonary hemorrhage. This species has been reported to produce penicillins, omega-hydroxy-emodin, roquefortin and xanthocillin. *Penicillium verrucosum* (species producing ochratoxin A, citrinin; isolate FRR 965) was from the Australian Culture Collection, Food Research Lab. *Paecilomyces variotti* (species reported to produce variotin and byssochlamic acid) was

isolated from a Cleveland house with a moisture problem; *Paecilomyces lilacinus* (species producing paecilotoxins; isolate 10114) was from the American Tissue Type Collection and *Aspergillus ochraceus* (species producing ochratoxin; isolate NRRL 398) was obtained from USDA Peoria Culture Collection. These species were selected as examples of potentially toxigenic fungi producing toxins other than the trichothecenes.

All fungi were cultured on potato dextrose agar for three weeks. The spores were harvested by gently wiping the surface of the cultures with a cotton swab, suspended in phosphate buffered saline and enumerated in a hemocytometer. Spores were characterized with respect to their cytotoxicity (MTT test using PK 15 porcine kidney cell line), proteolytic activity (fluorescence based EnzCheck assay using gelatin as a substrate; Molecular Probes, Eugene, OR), viability (dilution plating) and hydrated size (microscopy). Spore volumes were calculated using either the formula for an ellipsoid or a sphere according to their shape.

Seven d old rats were exposed via tracheostomy to 2×10^5 spores/gm BW of different fungi (8 pups/group). Two doses were used for *S. chartarum*. The animals were sacrificed 48 h later, bronchoalveolar lavage (BAL) was performed or the lungs were processed for histological examination. Control animals (n=8) received PBS.

The dose of 2×10^5 spores/gm that leads to measurable inflammation was selected based on our earlier studies of different doses of *S. chartarum* on the lungs of infant rats. Time period of 48h post exposure was selected based on earlier experience with infant rats showing significant increases in inflammatory indices within 72h corresponding to the period of acute lung injury (Yike, Dearborn, 2004). Additional set of animals not undergoing bronchoalveolar lavage was used for histopathological evaluation of the lung tissue. The levels of different cell types in the BAL fluid (BALf) were determined based on the counts of total leukocytes and erythrocytes in the BALf and differential cell counts using cytopspin slides. Total protein in the BALf was measured by the method of Bradford (Biorad, Hercules, CA) and proinflammatory cytokines IL-1 β and TNF- α were quantified by ELISA (R&D Systems, Minneapolis, MN; detection limit 5 pg). The final concentration of the cells and proteins in the BAL fluid was calculated per milliliter of the epithelial lining fluid using the urea dilution factor to correct for the dilution of the BAL fluid.

All data are expressed as the mean \pm SEM. All statistical tests (ANOVA followed by Dunn's test) were performed using Sigmastat version 2.03 (Jandel Scientific, San Rafael, CA) and the results were considered statistically significant at <0.05 probability level.

RESULTS

Characterization of fungal spores: Fungal spores collected from the cultures of *S. chartarum* and five other species differed in size and shape, viability, cytotoxicity and the ability to hydrolyze proteins. It is evident that the dimensions of the spores of *S. chartarum* are significantly larger than those of other species studied; their volume is on the average at least five times higher (Table 1). In order to correct for this difference an additional, reduced dose of *S. chartarum* (40,000 spore/gm) was used in the animal experiments.

Table 1. Cytotoxicity and proteinase activity of fungal spores.

Fungus	Cytotoxicity activity EC50 (spores/ml)	Gelatinase units/10 ⁶ spores	Hydrated spore volume (μm ³)
<i>S.chartarum</i> 58-17	70,000	1.35	100.0
<i>S.chartarum</i> 58-06	5,774,000	3.32	100.0
<i>Aspergillus ochraceus</i>	12,080,000	0.12	11.1
<i>Paecilomyces lilacinus</i>	300,000,000	0.20	7.0
<i>Paecilomyces variotti</i>	1,000,000,000	0.01	10.0
<i>P.chrysogenum</i> 1	>1,000,000,000	0.16	21.2
<i>P.chrysogenum</i> 7	>1,000,000,000	0.09	21.2

The ranking of the fungal isolates by their cytotoxicity to PK15 cells is shown in Table 1. Two isolates of *S. chartarum* differ in their cytotoxicity 83-fold. The toxicity of *S. chartarum* JS58-17 is at least 1.4×10^4 times higher than that of *P. chrysogenum*, *P. verrucosum* and *Paecilomyces variotti* and 4.3×10^3 times higher than that of *Paecilomyces lilacinus*. *Aspergillus ochraceus* was the second most toxic species (~173 times less toxic than *S. chartarum*).

The proteolytic activity of spore extracts calculated per 1 mln spores was also the highest for *S. chartarum* and also different for the two isolates, although *Paecilomyces lilacinus* had comparable proteolytic activity on a volume of spore basis.

The viability of the spore preparations obtained under identical growth conditions was variable, with high values for *S. chartarum* (>80%) and *P. chrysogenum* (69 and

55%) and very low values for *Penicillium verrucosum* (3%) and *Paecilomyces variotti* (6.55%).

Growth and survival of animals exposed to fungal spores: All rat pups exposed to 2×10^5 spores/gm of *S. chartarum* JS 58-17 died within the first 24 hours. 100% of the animals from other experimental and control groups survived, did not show any signs of distress and appeared to be healthy. Only the pups exposed to *A. ochraceus* (P=0.023) and *S. chartarum* JS 58-06 at 200,000 spores per gram (P=0.001) showed statistically significant reduction in their weight compared to PBS treated controls.

Pulmonary effects of exposure to fungal spores: Histological evaluation of the lung sections obtained from the animals treated with fungal spores shows signs of inflammation such as increased numbers of macrophages, neutrophils, lymphocytes and erythrocytes in contrast to the lungs of PBS treated control rat pups. Fungal spores observed in alveoli, often appeared to have been ingested by macrophages. The inflammation appeared to be most severe in animals treated with two isolates of *S. chartarum* while it was relatively mild in animals treated with other fungi.

Macrophages were significantly elevated in the BAL fluid of rat pups exposed to 200,000 spores/gm of *S. chartarum* JS 58-06 (1.4×10^7 cells/ml, P=0.043) and 40,000 spores/gm of *S. chartarum* JS 58-17 (1.6×10^7 cells/ml, P=0.016). In animals exposed to *A. ochraceus* the concentration of macrophages was only about half of that observed in the animals treated with PBS (2.5×10^6 cells/ml, P<0.001).

Neutrophils were rare in the BAL fluid from control animals ($\sim 4 \times 10^4$ cells/ml) and from rats exposed to *A. ochraceus* ($\sim 1.3 \times 10^5$ cells/ml). They were elevated to 0.8×10^6 - 5.1×10^6 per milliliter of epithelial lining fluid in animals exposed to the spores of indoor molds other than *S. chartarum* (Fig.1). The levels observed after treatment with *S. chartarum* were much higher exceeding 2×10^7 cells /ml for 200,000 spores/gm of JS 58-06 and reaching about 1.0×10^7 in animals exposed to 40,000 spores/gm of both JS 58-06 and JS 58-17. The numbers of neutrophils in the BAL fluid from animals treated with *S. chartarum* were all statistically increased (P < 0.03) over those treated with other fungi. Significant, several-fold increases in the BAL fluid lymphocytes were observed in nearly all treatment groups. Exposure to *Aspergillus ochraceus* led to almost 5-fold reduction ($\sim 4 \times 10^3$ cells/ml ELF; P=0.045) in BAL lymphocytes. The highest increases in the number of erythrocytes were noted in the BAL fluid from animals treated with *P. chrysogenum* 1 (~ 3 -fold, P<0.001) and *P. variotti* (~ 4 -fold, P=0.003).

Changes in the concentration of erythrocytes in the animals treated with *S. chartarum* were not statistically significant.

The concentration of total protein in the BAL fluid was significantly elevated ($P < 0.05$) in all animals exposed to fungal spores except for those treated with *A. ochraceus* and *P. verrucosum*. The highest increases were observed for *S. chartarum* isolate JS 58-17 (3-fold, $P < 0.001$).

Figure 1. Neutrophils in the BAL fluid from infant rats exposed to fungal spores. Values are mean \pm SE (n=8); SC - *S. chartarum*.

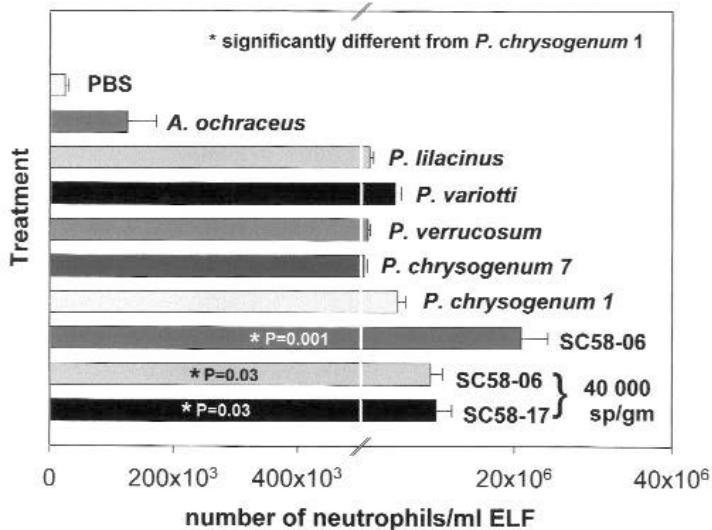
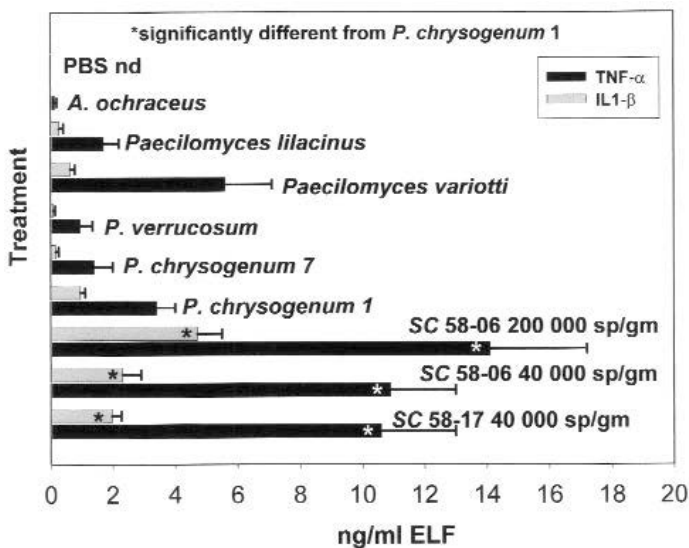


Figure 2. Proinflammatory cytokines in the BAL fluid of infant rats. Values are mean \pm SE (n=8); nd - not detected; SC - *S. chartarum*.



No proinflammatory cytokines could be detected in the BAL fluid from control animals (Fig.2). Trace amounts of TNF- α were measured in animals exposed to *A. ochraceus*. Low levels ranging from 3-6 ng/ml of epithelial fluid were detected in animals exposed to *Penicillium* and *Paecilomyces*. The levels of TNF- α in animals treated with *S. chartarum* exceeded 10 ng/ml and were statistically increased ($P \leq 0.02$) over those obtained after treatment with other fungi. Interleukin 1- β was detected in animals exposed to all fungi except for *A. ochraceus*. Similarly to TNF- α , the levels of IL-1 β were the highest in animals exposed to both doses and strains of *S. chartarum* and differed significantly ($P < 0.001$) from those observed for *P. chrysogenum* 1.

DISCUSSION

Fungal spores obtained from different molds under identical growth conditions showed different levels of viability, cytotoxicity and proteolytic activity. This, in addition to different sizes of fungal spores, makes the proper standardization of spore preparations virtually impossible and complicates direct quantitative comparison of their *in vivo* effects.

Normalizing the spore preparations for β -D-glucan or protein content would result in large differences in the volume of spore load instilled. Because clearance by macrophages is highly dependent on the volume of instilled particles, spore volume may be the most appropriate normalization factor for comparing different spore preparations in the lung although loads with identical volume will still vary greatly in their composition and metabolic activity, i.e. ability to release active proteins due to differences in viability. An attempt on standardization has been made by introducing the second, lower dose of *S. chartarum* (40,000 sp/gm compared to 200,000 sp/gm) which brings down the total volume of instilled *S. chartarum* spores closer to the range of that of other fungi (see Table 1).

Earlier studies conducted in our laboratory and those of others (Yike, Dearborn, 2004) indicated that the severity of pulmonary effects of *S. chartarum* seemed to be related directly to trichothecene toxicity. More recent data suggest that other spore components such as proteinases and hemolysin also contribute to the development of inflammation and lung injury, and that the effects of β -D-glucan are relatively mild. The spores of *S. chartarum* not only show the highest mycotoxin cytotoxicity but also contain higher levels of proteinases and have the highest viability. While higher doses of satratoxin producing isolate JS58-17 were lethal, strong responses elicited by the less toxic JS58-06 confirm the importance of both mechanisms in the pathophysiology of spore inhalation. The finding that highly viable isolate JS58-17 at 200,000 spores/gm BW caused 100% mortality is in deference to the same dose of non-viable spores used in our earlier studies (Yike, Dearborn, 2004)

and suggests that spore viability in addition to mycotoxins contributes to the pulmonary pathophysiology.

Even when a reduced dose of *S. chartarum* was used to correct for differences in spore size and the volume of the instilled spores was matched to that of *Penicillium spp.*, many of the effects of *S. chartarum* on the lungs of infant rats were significantly larger compared to other indoor molds. BAL fluid neutrophils and cytokines that could not be detected in control animals appear to be the most sensitive indicators of inflammation resulting from exposure to fungal spores.

The suppressed inflammatory responses and impaired growth of infant rats exposed to cytotoxic *A. ochraceus* may be related to the presence of ochratoxin which has been observed to be immunosuppressive (Bondy, Pestka, 2000).

CONCLUSIONS

The high impact of the spores of *S. chartarum* on the lungs of infant rats appears to correspond to their high cytotoxicity, proteolytic activity and viability. While the trichothecene production by this species remains the main concern, inhalation exposure to the isolates of *S. chartarum* that do not produce satratoxins and to other indoor molds may lead to injury in developing lung.

ACKNOWLEDGEMENTS

This work was supported by NIEHS grant ES09742.

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Chapter 5

Assessment I – Assessment Methods and Field Findings

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EXPOSURE IN MOLDY BUILDINGS

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INTRODUCTION

The association between moisture and microbial growth and adverse health effects among the occupants has been shown in a number of epidemiological studies (Bornehag *et al.* 2001), but the causal connections between various exposing agents and the health endpoints are not well known. The different health effects probably have different causative agents and they may develop via different mechanistic pathways. At present, a number of different parameters can be used as surrogates of the moisture and mold-related exposure, but more precise knowledge on the dose/response relationships would be needed for systematic risk assessment.

AIRBORNE FUNGI

Fungal growth or mold can usually be visually observed in moisture damage situations, and thus fungal spores have been the primary candidates for being the causal factors of reported respiratory symptoms. In the present literature, however, there is no conclusive evidence that increased concentrations of fungi in indoor air would be the actual cause for the symptoms (Verhoeff, Burge, 1997). In some studies, the concentrations of fungi in moisture damaged indoor environments have been somewhat higher than in normal conditions (Hyvärinen *et al.* 1993; Waegemakers *et al.* 1989) but the indoor concentrations are often much lower than those in outdoor air during the frost-free seasons. The levels may remain low even when visible mold is present (Miller *et al.* 2000). On the other hand, the indoor fungal concentrations have a high spatial and temporal variation, and the concentration distributions of moldy and normal indoor environments are largely overlapping (Hyvärinen *et al.* 2001).

The normal mycoflora of indoor air consists mainly of outdoor air genera such as *Penicillium*, *Cladosporium*, *Aspergillus*, yeasts and non-sporing isolates, but unusual genera or species may occur in a case of a moisture damage (Miller *et al.* 2000). Genera or species that typically grow on moisture damaged building materials are e.g., *Aspergillus versicolor*, *Aspergillus penicillioides*, *Stachybotrys chartarum*, *Chaetomium sp.*, *Fusarium sp.*, *Trichoderma sp.*, *Acremonium sp.*, and among bacteria, streptomycetes. However, the overall diversity of fungi and bacteria that have been detected in

buildings and moisture damaged materials is remarkable (Hyvärinen *et al.* 2002; Shelton *et al.* 2002).

OTHER AGENTS

Viable fungi only represent about 1% of the airborne fungal particles actually present in an indoor environment (Toivola *et al.* 2002). Therefore, the microbial load of indoor environments has also been described with enzymatic methods or chemical markers of biomass. Structural components of microbial cells, such as endotoxins of gram negative bacteria, 1,3- β -glucans of fungi and some bacteria, and ergosterol of filamentous fungi are possible parameters to determine (Park *et al.* 2001; Rylander, 1997; Dharmage *et al.* 2001). However, conclusive results on whether these parameters correlate with health effects or could be regarded as actual causal agents of symptoms in moisture damaged indoor situations have not been shown.

Volatile organic compounds, VOC, are involved in many occasions of biological or chemical decay induced by moisture. Some compounds originate from microbial metabolism (Korpi *et al.* 1998). Although the odor of mold is associated with symptoms, the exact role of VOC exposures as causal agents of irritation symptoms is still obscure.

Microbial toxins are potentially toxic substances produced by the secondary metabolism of many microbial species occurring on moldy building materials. The occurrence of toxins in the spores has been shown (Croft *et al.* 1986), and this may be the pathway how they may end up in the indoor air, and possibly in the human airways. However, indoor exposure to microbial toxins via airborne route is still insufficiently known.

PERSONAL EXPOSURE VS. STATIONARY SAMPLING

Exposure to various biological agents has usually been described with indirect methods, for example, as presence or absence of an agent in an indoor environment, or as concentration of an agent in settled floor dust. There is only limited data on actual measured exposures to biological agents on individual level. In a recent study, personal exposures to microbes of 81 teachers were compared with the results of stationary sampling (Toivola *et al.* 2002). Two 24 h measurement periods were used for personal sampling, and stationary samples were collected in homes (16 h) and workplace (8 h). Personal exposures were higher than the concentrations measured with stationary sampling, indicating the effect of the so called personal cloud, resulting from movements of the individual.

CONCLUSIONS

The exposure in moldy indoor environments is a complex issue, and little causal connections to health effects have been shown so far. It is evident that more detailed work is needed to describe the source, i.e., the microbial ecology of moistened building materials; the emissions from the sources, and the spatial, timely and size distributions of the various agents in indoor environments before the characteristics of the exposure are understood.

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DEVELOPMENT OF A GEL-TRAP SYSTEM COUPLED WITH PCR FOR MICROORGANISMS DETECTION: APPLICATION TO MYCOBACTERIUM TUBERCULOSIS COMPLEX

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ABSTRACT

The aim of this work was to develop a gel-trap system coupled with PCR in order to capture and to detect biocontaminated particles in indoor environments. We used this approach for the study of *Mycobacterium (M.) tuberculosis* complex in critical environments: hospital rooms with tubercular patients and a specialized laboratory for Mycobacteria culture.

The gel-trap system is placed in petri dish instead of traditional media and used in different manners: (i) with a multiple-hole impactor for air sampling, (ii) as passive sampler, it is positioned horizontally or vertically, in order to capture particles by sedimentation and diffusion, (iii) lastly, it is employed for contact surface sampling. The detection of captured *M. tuberculosis* complex is realized by PCR with a high control of taq polymerase environmental airborne inhibitors. Samples were taken during 2 or 3 minutes for air and during 2.5 hours to 8 days for sedimentation/diffusion. In 8 hospital rooms with tubercular patients, 42 sedimentation samples, 27 diffusion samples and 16 air samples were obtained. *M. tuberculosis* complex was detected in 3 rooms, 5 times with sedimentation gels, 2 times with diffusion gels and 1 time with the multiple-hole impactor. In the specialized laboratory, 2 sedimentation samples were positive on 26 tests carried out on the whole (21 sedimentation gels, 3 contact gels and 2 air samples).

This study shows that the gel trap system coupled with PCR allows the detection of *M. tuberculosis* complex, a slowly growing pathogenic bacterium usually difficult to identify in the environment.

INDEX TERMS: *Mycobacterium tuberculosis* complex, PCR, Microorganisms environmental detection, PCR, Hospital

INTRODUCTION

Indoor bioaerosols monitoring meets with many different problems, including: (i) culture sampling duration is limited in order to avoid the stress of microorganisms, and represents a difficulty for exposure assessment, (ii) bioaerosols belong to the particulate field and their composition varies in time and space (iii) lastly slowly growing microorganisms are rarely identified with classic sampling and culture methods (Griffiths, 1994).

The aim of this study was to develop a gel-trap system to capture microorganisms in various environments, without time limitation of sampling, and to analyze the product by polymerase chain reaction (PCR).

First of all, PCR was applied for the detection of *Mycobacterium tuberculosis* (Mt). In many developed countries, incidence of Mt infections in big cities remains high. For example, in Paris the 46,3 cases per 100,000 habitants correspond to 4 times the French national average (Decludt, 2001). This high score is mainly attributed to the worsening of the social and economic conditions for a part of the population excluded of health care system (Gutierrez, 1998). A reliable method to detect Mt spreading would be useful to survey environment (air, surface) biocontamination in risk area and to limit infection risk of people (hospital and social assistance personnel) surroundings patient with chronic pulmonary tuberculosis. Although investigators have successfully aerosolized and sampled mycobacteria in laboratory conditions (Loundon, 1969; Riley, 1976) (there is no more recent study on this topic to our knowledge), attempts to culture the mycobacteria from airborne particles, or droplet nuclei, expelled from the human respiratory tract failed, resulting in no growth or fungal overgrowth (Macher, 1992). The detection of airborne Mt can not be made using standard bioaerosol sampling methods. On the other hand, previous studies have successfully combined air sampling by filtration and PCR to detect aerosolized Mt in laboratory conditions (Schafer, 1998, 1999).

The present study coupled air, sedimentation and surface gel-trap collections followed by Roche Amplicor® PCR detection of MT complex. The commercial Roche Amplicor® Mt PCR kit was adapted to eliminate PCR inhibitors in samples collected from the airborne environment, permitting detection of Mt DNA. We evaluated the detection limit of the system gel-trap-PCR using *Mycobacterium bovis* BCG (Bacillus of Calmette-Guérin) as a Mt surrogate. The method was assessed on-site in hospital rooms welcoming tuberculosis patients and in a laboratory specialized in mycobacterial culture.

METHODS

Mycobacteria culture: *M. bovis* BCG strain (ATCC 35734) was used to evaluate the sensitivity of the gel-trap-PCR system. Bacteria were cultivated on Colestos agar medium during 4 weeks at 37°C.

BCG homogenous suspensions in PBS were calibrated using a spectrophotometer (λ : 620 nm). Confirmation of the bacilli concentration was obtained by successive dilutions in PBS and 100 μ l samples were plated into complete Middlebrook (7H10 + 10% OADC) agar medium. Colonies were counted after 4 and 6 weeks of incubation at 37°C.

Sample collection: The gel-trap composed of Low Melting Point agarose 0,2 % (wt/vol), glycerol 10 % (vol/vol) and sterile water for injection is deposited in sterilized Petri dishes. The gel-trap has the capacity to capture airborne particles and to remain moisturized during long periods (several weeks).

Different modes of sampling were used: airborne particles were collected (i) with a multiple-hole impactor AT-OMEGA® (flow rate:100 l.min⁻¹) (ii) by sedimentation or diffusion on Petri dishes respectively disposed horizontally or vertically (iii) after sedimentation of particles, by contact surface sampling with the gel-trap

Sample pre-treatment: At the end of the sampling period, gels were collected, liquefied by heating, centrifuged and transferred into sterile 50 ml screw-cap tube filled with 20 ml of sterile water. After 5 minutes at 90°C (water bath) a liquid consistency was obtained. Each sample was centrifuged twice at 3,000 x g for 15 minutes and pellets containing airborne particles were resuspended in 200 μ l of sterile water and stored at - 20°C.

PCR detection: The Roche Amplicor® Mt test (Roche Diagnostic Systems Inc) routinely employed to detect Mt complex in clinical specimens was used according to the manufacturer's instructions. Briefly the amplification of a sequence of 584 nucleotides in the gene coding for the 16S rRNA (one target per cell) is realized by the use of KY18 and KY75 primers, specific of the Mycobacterium genus. Then, *M. tuberculosis* complex is detected by the hybridization of a species-specific probe selected from the hyper variable region of the 16S rRNA gene.

As PCR inhibition can induce false-negative reactions, we incorporated internal controls in each reaction tube. To neutralize PCR inhibitors from environmental samples and to obtain a sensitive method we added two-steps to the original test: a filtration (4nm Millex, Millipore) and an addition of Bovine Serum Albumin (BSA, Sigma) in PCR mixture before amplification.

RESULTS

Gel-trap-PCR system sensitivity: We performed the detection of suspensions of *M. bovis* BCG going from 5.10^7 to <1 colony forming unit (CFU) per sample, in presence of the gel-trap or in PBS as control.

Five independent experiments were conducted in presence or in absence of PCR inhibiting airborne environmental particles. The addition of BSA ($8 \mu\text{g}/\mu\text{l}$) and the filtration step were performed before amplification.

Table 1. Detection limit of the gel-trap-PCR system

<i>M. bovis</i> BCG CFU/sample	PCR detection (%)			
	without inhibiting airborne environmental particles		In presence of inhibiting air- borne environmental particles	
	PBS	Gel-trap	PBS	Gel-trap
5.10^4	100	100	100	100
5.10^3	100	100	100	100
5.10^2	100	100	100	20
50	100	100	100	0
5	100	0	80	0
0	0	0	0	0

Table 1 confirms the sensitivity announced by the Amplicor manufacturer. The use of gel even with BSA and filtration gives a loss of 2 log of sensitivity (5.10^3 cfu/sample).

In use assays in tubercular patients hospital rooms: Patients were selected after preliminary clinical diagnosis of tuberculosis pneumonia based on acid- fast bacilli (BAAR) microscopy in sputum samples (BAAR determination/microscope field). All patients were placed in single isolation room naturally ventilated.

Table 2. Mycobacterium tuberculosis detection with the gel-trap-PCR system in hospital rooms of tubercular patients

patient	gender	age	BAAR per field	Mycobacteria isolated	duration (days) sedimentation/ diffusion samples ¹	air volume AT-Omega samples	samplings number ²	positive PCR
A	M	29	1-9	tuberculosis	6	2x1m ³	(i) = 11 (ii) = 4 (iii) = 2	1 0 1
B	M	72	>100	africanum	-	2x1m ³	(i) = 0 (ii) = 0 (iii) = 2	- - 0
C	F	30	>100	tuberculosis	5	2x1m ³	(i) = 4 (ii) = 4 (iii) = 2	0 0 0
D	M	39	<1	tuberculosis	4	2x1m ³	(i) = 4 (ii) = 4 (iii) = 2	0 0 0
E	M	30	<1	tuberculosis	5	2x1m ³	(i) = 4 (ii) = 4 (iii) = 2	0 0 0
F	M	45	>100	tuberculosis	7	2x1m ³	(i) = 3 (ii) = 4 (iii) = 2	3 2 0
G	M	27	>100	tuberculosis	7	2x1m ³	(i) = 7 (ii) = 2 (iii) = 4	0 0 0
H	M	26	20	tuberculosis	7	-	(i) = 9 (ii) = 5 (iii) = 0 (iv) = 2	1 0 - 0

¹ “-” : not concerned

² Sampling mode: (i) = sedimentation; (ii) = diffusion; (iii) = impaction; (iv) = contact

Mt complex was detected in 3 rooms, 5 times with sedimentation gels, 2 times with diffusion gels and 1 time with the multiple-hole impactor. The environmental detection is not correlated with the number of BAAR detected in sputum samples during the sampling period.

In use assays in a hospital laboratory: Environmental sampling were also done in a hospital laboratory specialized in culture and identification of *Mycobacteria*, in various locations: the laboratory itself, the microbiological safety cabinets and the incubation room.

Table 3. Mt* detection with the gel-trap-PCR system in the laboratory

site	duration sedimentation/diffusion samples	air volume AT-Omega samples ¹	samplings number ²	positive PCR
laboratory	2h30 – 8 days	-	(i) = 8	0
safety posts	8 days	-	(i) = 3	0
incubation room	2h30 – 8 days	3 m ³	(i) = 10 (iii) = 2 (iv) = 3	2 0 0

¹ “-” : not concerned

² Sampling mode: (i) = sedimentation; (ii) = diffusion; (iii) = impaction; (iv) = contact

Two sedimentation samples (table 3) were positive in the incubation room near the door.

DISCUSSION

The major obstacle in using PCR for environmental samples is the presence of inhibiting environmental particles (i.e. phenolic compounds and humic acids in water, dust and pollen in air...) collected at the same time as the required germ (Alvarez, 1995). The PCR failure may be total or partial, the biological mechanism of the reduced sensitivity being not well understood (Wilson, 1997).

In order to reduce the effect of inhibitors from airborne environmental samples obtained in the presented study two steps were added to the conventional protocol: a BSA addition and a filtration just before amplification. The positive effect of BSA previously shown (Loomis, 1974) was confirmed in our study in a constant way in spite of variability of inhibitors collected in the course of time. BSA is able

to bind to many molecules and may capture inhibiting substances not retained at the time of the filtration step.

By using the gel-trap system, a factor of sensitivity of about 100 is lost if compared to the use of PBS. But recent assays showed that the simple modification of the heating conditions (1 hour at 62°C instead of 5 min at 90°C) allows the gain of a factor of sensitivity of about 10, probably by allowing a better depolymerization of the agarose chains.

The on-site assays showed the interest of the gel-trap-PCR system to characterize the contamination of an indoor environment by Mt without culture. The presence of treated patients (e.g. no MT bacilli emitted) or opening of windows during strong summer heats without protection against outdoor pollutants may explain that the detection is not constant. When Mastorides *et al.* in 1999, successfully detected Mt by PCR in the air of isolation rooms occupied by tubercular patients, the air was treated with HEPA filters expected to remove 99,9% of airborne pollutants.

Positive results obtained in the incubation room of the specific laboratory can be explained by the fact that Mt smears for further microscopic observation were put in to dry with the free air near a ventilator. After correction of this bad practice, the search for Mt by PCR proved to be negative

IMPLICATIONS

The gel-trap-system may provide a good approach to study the indoor environments potentially contaminated by other biological agents difficult to detect by traditional culture methods.

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HOUSE DUST: AN EFFICIENT AND AFFORDABLE TOOL TO ASSESS MICROBIAL CONTAMINATION IN HOMES

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ABSTRACT

In Greater Montreal, in 2000 and 2001, the microbial contents of dust from 68 “healthy” houses without water damage or health complaints from occupants was compared to that of 145 “sick” houses with significant water damage. Mean fungal counts were 7 times higher in the sick homes. The fungal distribution in healthy houses, where *Cladosporium*, *Alternaria* and other phylloplane species predominated, differed from that of unhealthy houses, where species of *Penicillium* and *Aspergillus* were predominant. These results demonstrate that the fungal content in dust is indicative of the microbial contamination of a house.

INTRODUCTION

In recent years, the family home has often become a source of microbial contamination, where molds, yeasts and bacteria proliferate. While there has been an increase in various allergy cases, pediatric asthma, atopy and hypersensitivity in North America (CDC Report on Asthma, 2000), this microbial contamination of the indoor environment is associated more and more often with health complaints from occupants (Pasanen, 2001).

Water damage episodes and excess humidity often cause the proliferation of fungi (molds), yeasts and bacteria on visible surfaces or hidden inside structures. In such a context, the importance of a good diagnosis of the degree of microbial contamination in homes becomes more important. Unfortunately, assessment tools are few and insufficient. Air sampling, still frequently used, is an incomplete tool, which is not reproducible and can lead to false negative results. On the other hand,

surface samples are useful to document the nature of visible fungal contamination but insufficient to obtain a global diagnosis (ACGIH, 1999).

Our field experience has led us to promote the use of house dust microbial analysis, as it represents the “memory of a building” giving valuable information on its microbial history (Miller, 1988; Wickman *et al.* 1992; European community, 1993; Flannigan *et al.* 1994; American Industrial Hygiene Association, 1996; Pasanen *et al.* 1997; Veerhoeff, Burge, 1997; Dillon *et al.* 1999; Hodgson, Scott, 1999; Mainville *et al.* 1999; Miller *et al.* 1999). This project was conducted to highlight the link between the history of water damage in a dwelling and the microbial content of the dust in that dwelling. We have already analyzed the microbial content of fungi, yeasts and bacteria, in dust samples taken from hundreds of problem dwellings. We also analyzed the microbial content of dust from more than fifty healthy homes, thus supplementing our database to confirm the validity of the method.

METHODS

Healthy home selection: Homes in the Montréal area were recruited through an advertisement placed in two newspapers (La Presse and Voir), direct faxing, door-to-door distribution of a brochure and word of mouth. A selection was made using a telephone questionnaire to eliminate homes that did not meet the criteria established for this project, including the following:

- no major water damage episode (s) lasting at least 24 hours, or chronic, during or since the 1998 ice storm ;
- no apartments located in basements ;
- no health problems having appeared or worsened since moving in after at least two years of occupancy ;
- no carpets in basements ;
- no poorly maintained forced air systems, with porous insulation or humidifiers.

Inspection protocol: Selected homes, from the telephone questionnaires, were thoroughly inspected by microbial indoor air quality consultants. The inspections, looking for any signs of water damage, lasted a minimum of one hour and a half and consisted of visiting the premises thoroughly both outside and inside, checking the structures with a moisture meter, taking photographs and sampling dust. The inspector double-checked with the occupants the accuracy of their answers on the telephone questionnaire, to rule out any major water damage episode or health problems.

Dust sampling: For dust sampling, inspectors used a portable Hoover Portapak vacuum cleaner with disposable paper bags to take a composite sample of dry

deposited dust from the occupied rooms. To reduce outside influence, samples were not taken from the floor but higher: for example, on bookshelves, kitchen shelves, door frames, etc. Depending on the level of dust accumulation, the total sampling area in the dwelling could be anywhere between 1 and 2 square meters (precisely measured). Vacuuming lasted 5 minutes on each surface. After sampling, the vacuum cleaner bag was removed, sealed with adhesive tape and identified with a number. It was then placed in a tightly sealed ziplock bag where it was kept at 4 degrees C until put in culture, with a maximum delay of 6 days.

Microbial analysis of dust samples: Suitably diluted in sterile water, dust samples were plated on MEA Rose bengal culture dishes for mold and yeasts and on PYA for bacteria. Duplicates of total bacteria were counted under the dissecting microscope after 48 hours of incubation at room temperature. Duplicates of yeast and fungi were counted under the dissecting microscope after 7 to 14 days of incubation at room temperature, depending on their speed of sporulation. The molds were identified to the genus level, and to the species level in some cases.

Statistical analysis: ANOVA testing was performed to determine statistical significance of data sets exhibiting a normal distribution. Wilcoxon/Kruskall-Wallis testing, using medians, was performed instead on data sets with skewed distributions. However, arithmetical means are showed in every Table, because they are more illustrative of the data and easier to compare.

One dust sample was collected in most water damaged homes and all non water damaged homes. However, up to 3 dust samples were collected in a small number of the larger homes, with multiple water damaged locations. For a few homes, the data bank was incomplete. Tables of results, for that reason, might contain slightly variable numbers of analyzed samples.

RESULTS AND DISCUSSION

General characteristics of data bank homes:

Table 1. Extent of water damage in the data bank homes

EXTENT OF WATER DAMAGE	NUMBER OF HOMES
Low ¹	68 (25%)
Significant to extensive ²	145 (75%)
Extensive ³	82 out of 145 (31%)
Total	213

¹ Low: “healthy houses” (Water damage episode (duration): none or less than 24 hours; Water damage history: none or light)

² Significant to extensive: “unhealthy houses” (Water damage episode (duration): 24 hours and more, one week and more, or chronic water damage history: moderate to extensive)

³ Extensive (a subgroup of significant to extensive): “very unhealthy houses” Water damage episode (duration): one week and more, or chronic water damage history: extensive

Table 2. Season of inspection

INSPECTION SEASON	LITTLE WATER DAMAGE	SOME WATER DAMAGE	EXTENSIVE WATER DAMAGE
Winter	11 (16%)	28 (19%)	9 (11%)
Spring	15 (22%)	22 (15%)	11 (13%)
Summer	6 (9%)	45 (31%)	31 (38%)
Fall	36 (53%)	50 (34%)	31 (38%)
Total	68	145	82

Fungal counts, extent of water damage and season: Results from Table 3 show that dust from unhealthy homes contained up to seven times more mold spores than that of their healthy counterparts (Wilcoxon/Kruskal-Wallis $p < 0.0001$). These results confirm the Ontario Wallaceburg study (Miller *et al.* 1999) where fungal counts from dust sampled in 20 out of 400 homes, with the most extensive water damage episodes, were 10 times higher than fungal counts from the 20 homes without excessive water damage. Results in Table 3, however, related to a much higher number of samples, which allowed us to validate them statistically.

Other publications also link contamination levels in buildings and fungal contents in carpet dust (Hodgson, Scott, 1999) and in dust deposited on smooth surfaces (Mainville *et al.* 1999).

Table 3. Fungal counts in dust and extent of water damage

WATER DAMAGE EXTENT	SAMPLE NUMBERS	MEAN FUNGAL COUNTS IN DUST (CFUs/g of dust)	STANDARD ERROR
Low	71	74,366	143,396
Significant to extensive	184	447,837	89,138
Extensive	95	548,179	124,053

Furthermore, Table 4 shows that fungal contents of dust from all homes is not significantly influenced by season, and that there is no interaction between season and the extent of water damage. Seasonal influence could not be shown in this data set, partly because settled dust is accumulating in homes during variable time lapses, from days to several weeks, depending on sampling site and frequency of cleaning. Water damage alone makes a significant difference in fungal counts, confirming Table 3.

Table 4. Fungal counts in dust, extent of water damage and season

INFLUENCE FACTOR	TWO WAY ANOVA p value
Extent of water damage	0.0108
Season	>0.05
Extent of water damage and season	>0.05

Ratio of non phylloplane to phylloplane fungi in dust related to extent of water damage: *Cladosporium* and *Alternaria* are the phylloplane, and *Penicillium* and *Aspergillus* the non phylloplane genera found most frequently in the dust of the inspected homes, whatever their contamination levels. Table 5 shows that phylloplane fungi predominate more often in healthy homes, while non-phylloplane fungi predominate in unhealthy homes. The difference is highly significant (ANOVA p 0.00040 for phylloplanes and 0.012 for non phylloplanes).

Table 5. Fungal distribution in dust related to extent of water damage

EXTENT OF WATER DAMAGE	SAMPLES	PHYLLO-PLANE (<i>Clado</i> + <i>Alt</i>) (standard error)	NON PHYLLO-PLANE (<i>Pen</i> + <i>Asp</i>) (standard error)	NON PHYLLO-PLANETO PHYLLOPLAN- ERATIO
Low	67	45% (3.7)	31.6% (4.05)	0.70
Significant to extensive	189	30% (2.2)	45.5% (2.41)	1.51

Bacterial counts in dust and extent of water damage: Table 6 shows that mean counts in bacteria are more than twice as high in unhealthy homes compared to healthy homes, but the standard deviation is too high to confer statistical significance to these data (Wilcoxon/Kruskal-Wallis $p > 0.05$). Many factors can explain these findings, for example the presence of pets, cold water humidifiers or sump pumps with improper maintenance, the season of inspection, etc. In the data bank, there are not enough homes with each of these separate characteristics to allow us to perform a statistical analysis of the bacterial counts in dust. Inspection findings indicate however that all these factors do have an influence on counts of bacteria in house dust.

Table 6. Bacterial counts in dust and water damage extent

EXTENT OF WATER DAMAGE	SAMPLE NUMBERS	MEAN BACTERIAL COUNTS cfus/gram of dust	STANDARD ERROR
Low	68	678 088	522 444
Significant to extensive	171	1 414 664	329 455
Very unhealthy	95	1 504 579	442 010

CONCLUSIONS

This study confirms the reliability of house dust sampling as a complementary diagnosis tool for the assessment of microbial contamination indoors. Fungal taxa in house dust are a good indicator of water damage extent and represent the “microbiological memory” of houses. Dust from water damaged homes can contain up to seven times more mold than that of their non water damaged counterparts and the fungal distribution often shows a majority of non phylloplane species in these water damaged homes.

ACKNOWLEDGEMENTS

This project was funded by Canada Mortgage and Housing Corporation (CMHC), under the terms of the External Research Program.

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MICROBIAL INDOOR AIR QUALITY IN OFFICE BUILDINGS WITH CENTRAL AIR CONDITIONING INSTALLATIONS IN BELGIUM. AN EASY TOOL FOR A FUNGAL EVALUATION

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ABSTRACT

From 1996 to 1999, 166 surveys were conducted in office buildings equipped with Heating, Ventilating Air Conditioning (HVAC). Mesophilic (MesM) and xerophilic (XerM) molds incubated at 25°C, and thermophilic species (ThM) growing at 45°C, were evaluated in the offices, at three sampling areas: room air in the breathing zone and at the diffuser outlet in the offices, and outdoors, in front of the outdoor air intake. Centiles 5, 25, 50, 75 and 95 were calculated for different fungal groups to serve as a baseline for interpretation of results, which are presented on a graph. With this graph, a global picture of the airborne fungal quality was obtained, allowing visualizing trends and imbalances, and informing about a possible total contamination. To complete this primary diagnosis, the next step consisted in mold identification. For practical reasons, we distinguished 6 essential fungal groups: 1. Total *Cladosporium* (mainly outdoor contaminants); 2. Total *Aspergillus* and 3. Total *Penicillium* (mainly indoor air contaminants); 4. Total of other taxa; 5. Dominant taxa (species identification); 6. Toxigenic molds.

INDEX TERMS: Airborne molds, Indoor air, workplaces with HVAC

INTRODUCTION

In temperate climates, people generally work in office buildings equipped with a Heating Ventilation and Air Conditioning (HVAC) system where many complaints are regularly reported (Anderson, 1998). Among potential agents responsible for these illnesses and disorders, it is well known that molds are sometimes an important source of contamination in buildings and in HVAC's (Ahearn, 1997; McGrath, 1999; Gravesen, 2000; Burge *et al.* 2000). Correlations are described for some fungi and Sick Building Syndrome (SBS) (Cooley, 1998; Halonen, 2000), and also for

other pathologies as allergic rhinitis, asthma, allergic fungal sinusitis, allergic bronchopulmonary mycosis, and extrinsic allergic alveolitis (hypersensitivity pneumonitis) (Estoppey, 1993; Hodgson *et al.* 1998; Fink, 1998 a, b; Westergren, 1998; Nolard, 1999; Husman, 2000; Ebbejoj, 2002). Production of compounds and presence of mycotoxins in conidia may also induce adverse health effects (Reijula, 1998; Rylander, 1998).

It is thus important to have an easy and standardized method to evaluate the fungal level of *Indoor air* (IDA). In HVAC systems, the outside air is filtered, contrary to an open environment such as a dwelling. Quantitative reference values, percentiles for instance, can be calculated, keeping in mind that these levels are not linked with health risks. They should be used as a tool to compare results with those from similar buildings.

MATERIAL AND METHODS

For each air sampling location, 3 samples were systematically taken with an RCS+ from Biotest, 2 on strips filled with HS, 1 on a strip filled with DG18 (Chasseur, 2000).

Outdoor air was always sampled on the day of the survey, in front of the outside air inlet duct, and was considered as daily reference. In the offices, the air was sampled at breathing level (room air), and at the HVAC diffuser (diffuser air). All the selected buildings were equipped with HVAC systems. Percentiles were calculated after elimination of the non numeric data because of confluence and invading species (Smid, 1989). The occurrence of a taxon was defined as the percentage of strip with a minimum of 1 cfu/volume sampled.

RESULTS AND DISCUSSION

1. Outdoor air: seasonal variations and limit values: Seasonal variations of airborne molds are well-known (Nolard, 1997). This is why outdoor percentages indicated in Table 1 cannot be used as a reference for the day of the survey. Outdoor air sampling provides a range of results, especially about the maximum values obtained.

2. Indoor air: total indoor airborne fungal concentration: In Table 1, the total airborne mold concentrations recorded in 75% of the buildings (percentile 75) were generally lower than 63 cfu/m³, and were considered as normal and typical. High airborne fungal concentrations were detected in 5% (> percentile 95) of the buildings inspected. In these cases a further survey was required, more detailed, with complementary procedures.

Table 1: Fungal enumeration (cfu/m³) in Indoor air of offices with air-conditioning. (RCSplus, HS (modified) and DG18 media, at 25°C during 5 days of incubation

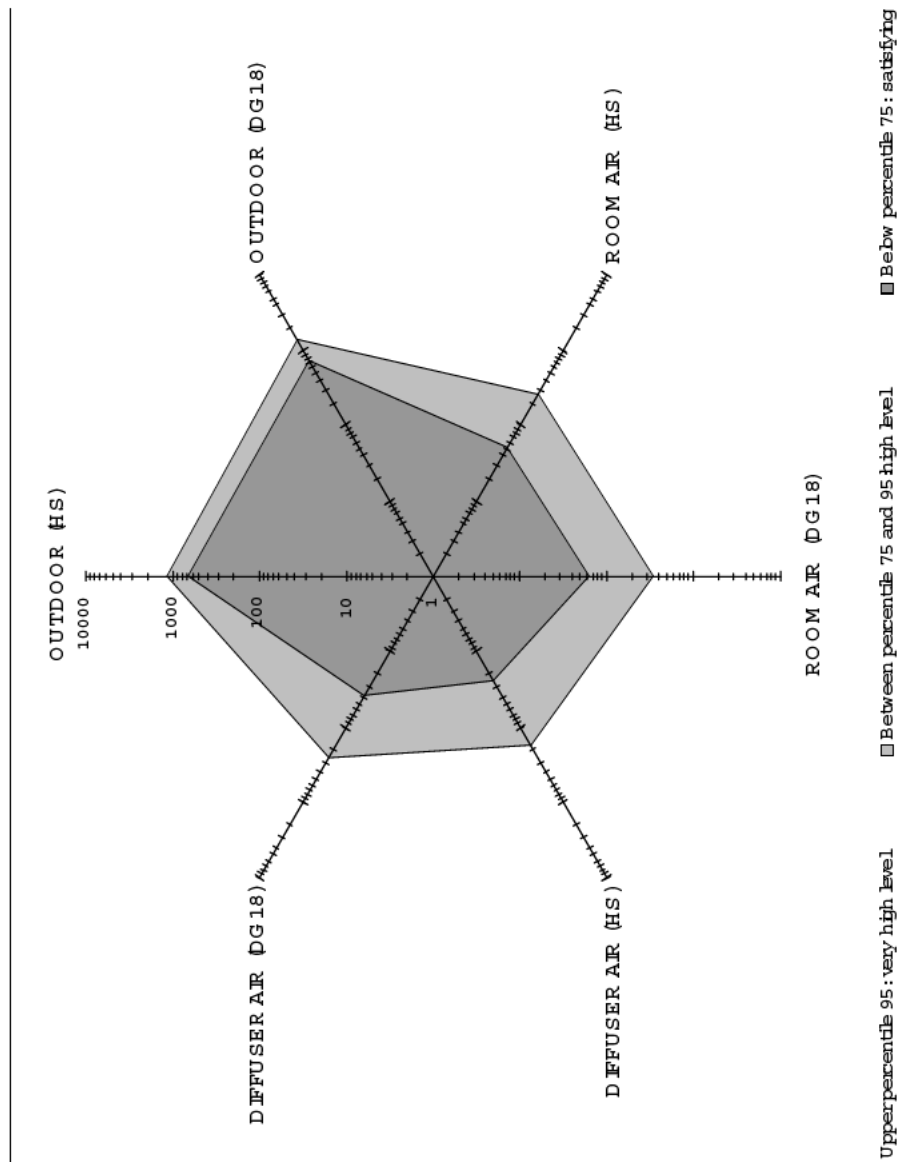
Percentiles	ROOM AIR		DIFFUSER AIR		OUTDOOR AIR ¹	
	HS	DG18	HS	DG18	HS	DG18
5	< 13	< 13	< 13	< 13	(38)	(88)
25	< 13	< 13	< 13	< 13	(125)	(200)
50	13	25	< 13	13	(250)	(388)
75	50	63	25	38	(663)	(713)
95	263	350	175	250	(1163)	(1325)
N ₀ (total data)	739	739	205	205	117	117
% conf & overgrowth	3.3	3.2	2.0	0.5	24.0	26.5
Min.	< 13	< 13	< 13	< 13	< 13	< 13
Max.	2225	2038	2225	1888	2225	> 2375

¹ Seasonal fluctuations, not a reference

3. Graphic representation of global results (Figure 1): To facilitate data interpretation, a graphic representation was plotted. Three fungal quality levels were delimited in the background by using increasing percentiles to serve as a baseline for interpretation: Normal and typical (below percentile 75), high (between percentiles 75 and 95) and very high (above percentile 95). Moreover, the results were reported on 6 axes, corresponding to mesophilic (*MesM*) and xerophilic (*XerM*) molds sampled in 3 strategic areas: outdoors during the survey and in workplaces (room air and diffuser air).

This preliminary evaluation was only quantitative, arranging the 3 categories of molds and the 3 defined areas in buildings. All the data were written on this graph to see the global “trends” of results in the different strategic places. The imbalances between the different categories should allow to point out some anomalies.

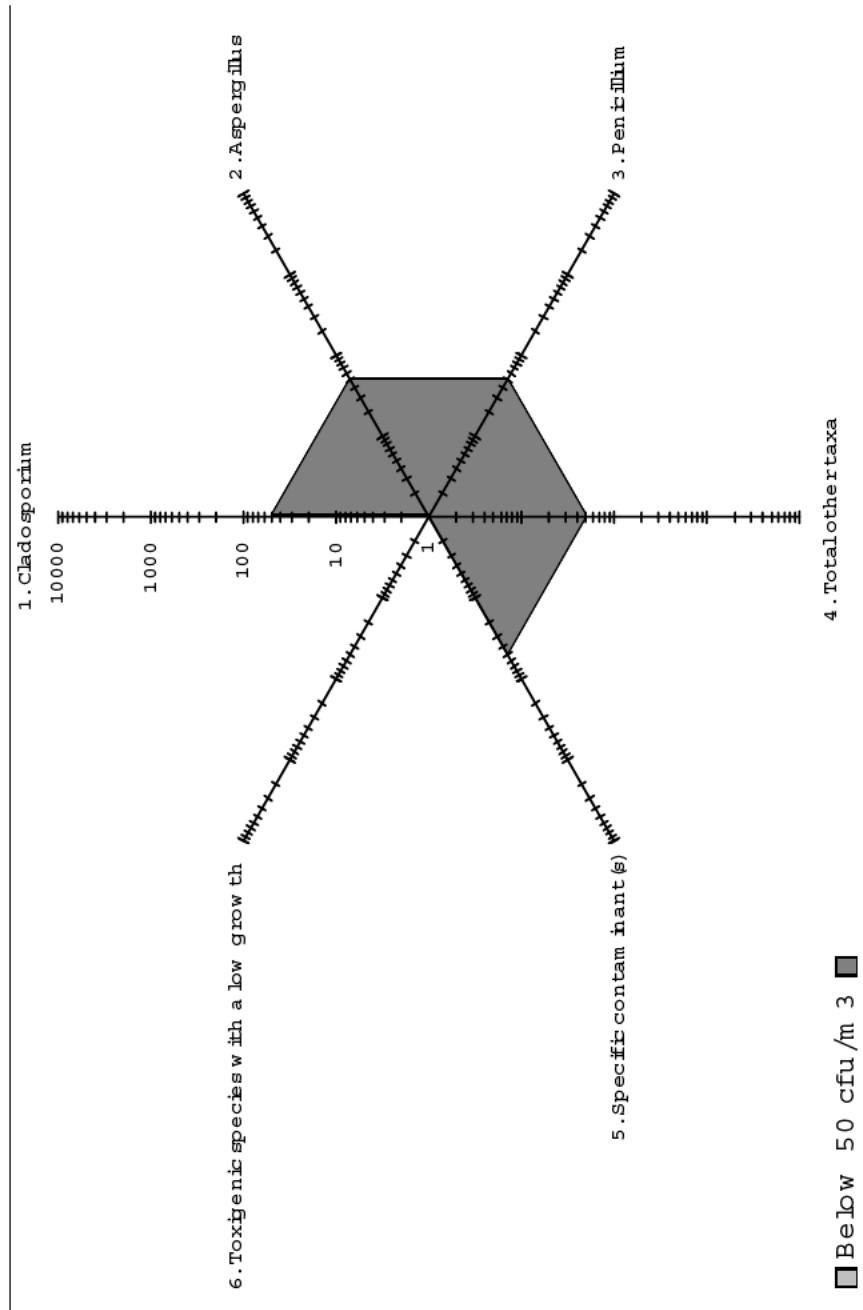
Figure 1. Graphic representation results obtained for mesophilic and xerophilic molds in buildings equipped with HVAC's (logarithmic scale, data in cfu/m³)



4. Importance of isolated species: a qualitative graphic representation (Figure 2): Identification to genus or species level is necessary, especially when Indoor airborne concentrations are rather high. It permits to distinguish outdoor from indoor air species and also to evaluate more precisely the specific risk for health. Moreover, the knowledge of mold ecology and spores spreading mode, helps to locate more accurately the source and cause of development, and to pro-

pose the most adequate remediation procedure. From the results (Table 2) we distinguished 6 essential fungal groups: Total *Cladosporium*, Total *Aspergillus*, Total *Penicillium*, Total of other taxa, specific dominant taxa and specific toxigenic molds.

Figure 2. Graphic representation results obtained for the 6 essential fungal groups in buildings equipped with HVAC's (logarithmic scale, data in cfu/m³).



Group 1 (Total *Cladosporium*): the genus *Cladosporium* is among the most common airborne fungi, and has a worldwide distribution. In Europe, three species are frequently isolated from air on selected media. *Cladosporium herbarum* is a very early colonizer on dying and dead plant substrate, leaves and stems in particular. Its presence in Indoor air in buildings is linked to seasonal variations (outside source): for example, deficient filters (high concentration at diffuser outlets) or windows opened just before the surveys (low concentration at openings). *Cladosporium cladosporioides* is also often considered as an outdoor source (on plant material and in soil) but indoor air amplification may occur (in humid cellars for instance). On the other hand, *C. sphaerospermum* is a species able to grow quickly on various damp material in buildings (paper, paint, textile, plaster) (Beguin, 1994; Nolard, 1999). In the buildings studied, about 80% of outside samples contained *Cladosporium spp.* (Table 3). This occurrence decreased in room air (32%) and even more at diffuser outlets (22%). Moreover, the maximum concentration obtained in indoors conditions (688 cfu/m³) were lower than the maximum results obtained in outdoor air (788 cfu/m³) (Table 5). *C. herbarum* was the most common species with an occurrence of 50% for outside, 9.5% for room air and 7% at the diffuser outlet, while *C. cladosporioides* and *C. sphaerospermum* were rarely recovered in this indoor environment. So, in HVAC systems, the *Cladosporium* genus may be generally considered as outdoor origin when present in high levels, and in this case, identification to the genus is generally sufficient.

Group 2 (Total *Aspergillus*): the genus *Aspergillus* contains a lot of species on a large scale of water activity and thermophilic characteristics. In Table 4, Total *Aspergillus* occurrence was higher outdoors with 42 to 50%, than in Indoor air (14 to 18% in room air and 10 to 15% at diffuser air).

The thermophilic species *Aspergillus fumigatus* presented the highest occurrence with 41% and 7% (incubated at 45°C) respectively in outdoor and indoor air. The xerophilic mold *A. glaucus gr.* also had a high outdoor occurrence on DG18, with 32%, for only 7% in room air. But, the maximum concentrations obtained in room air conditions (2225 cfu/m³) were higher than the maximum results obtained outdoors (113 cfu/m³) (Table 5). These results confirm the indoor origin of the *Aspergillus* genus when present in high levels due to amplification, or due to high accumulation of species thanks to a long viability of their conidia.

Table 2. Occurrence (%) of the main fungal genera in Indoor air of offices with HVAC systems. With RCSplus, HS (modified) and DG18 media, at 25°C during 5 days of incubation

Genus	HS			DG 18		
	Room n = 739	Outside n = 117	Diffuser n = 205	Room n = 739	Outside n = 117	Diffuser n = 205
<i>Acremonium</i> ⁽¹⁾	1,22	4,27	0,49	0,00	0,00	0,00
<i>Alternaria</i>	3,65	17,09	2,93	1,62	5,98	1,95
<i>Apiospora</i>	0,27	0	0	0,00	0	0
<i>Artbrinium (teleomorph Apiospora)</i>	0,27	0,86	0,49	0,14	2,56	0,00
<i>Aspergillus</i>	14,21	41,88	9,76	18,00	50,43	15,12
<i>Aureobasidium</i> ⁽¹⁾	0,14	0	0	0,27	0,85	0
<i>Botrytis cinerea</i>	4,87	27,35	4,88	2,30	11,97	1,46
<i>Botryotrichum</i>	0,27	0,86	0	0,00	0,00	0
<i>Cladosporium</i>	31,80	79,49	21,95	31,80	80,34	23,90
<i>Chaetomium</i>	0,27	0,86	0,49	0,00	0,00	0,00
<i>Exophiala</i> ⁽¹⁾	0	0	0	0	0	0
<i>Fusarium</i> ⁽¹⁾	0,27	0,86	0	0,00	2,56	0
<i>Geotrichum</i>	0	0,86	0	0	0,00	0
<i>Mucor</i>	0,54	0,86	0,49	1,08	0,85	0,49
<i>Paecilomyces</i>	0,27	0,00	0,49	0,41	0,85	0,49
<i>Penicillium</i>	23,82	64,10	16,10	26,52	75,21	19,02
<i>Phoma</i> ⁽¹⁾	0,41	0,86	0,00	0,54	0,85	0,00
<i>Rhizopus</i>	1,49	1,71	0	0,41	0,85	0
<i>Scopulariopsis</i>	1,22	5,98	0,49	0,68	0,85	0,49
<i>Stachybotrys (S. chartarum)</i>	0,14	0,86	1,46	0,00	0,00	0,00
<i>Trichoderma</i>	1,62	4,27	0,49	0,00	0,85	0,00
<i>Ulocladium</i>	1,49	8,55	3,90	1,08	1,71	1,95
<i>Wallemia (W. sebi)</i>	0,00	0,00	0,00	1,08	4,27	0,49
Others	2,71	8,55	2,44	2,03	2,56	0,00
White sterile mycelia	14,88	25,64	11,71	11,64	15,38	6,34
Gray sterile mycelia	3,79	5,13	2,44	14,48	14,53	10,73
Gray sterile myc. " <i>Cladosporium</i> like"	9,74	19,66	8,29	14,48	60,68	13,66
Yeasts	10,96	47,01	5,85	10,55	24,79	8,29
Confluence	1,08	21,37	1,46	2,30	29,06	1,46
Overgrowth	2,30	8,55	0,98	0,81	4,27	0

⁽¹⁾: often abundant in humidifier water

Table 3. Occurrence (%) of the main *Cladosporium* species

On HS	ROOM n = 739	OUTSIDE n = 117	DIFFUSER n = 205
<i>Cladosporium spp.</i>	31,80	79,49	21,95
<i>C. cladosporoides</i>	2,03	8,55	0,00
<i>C. herbarum</i>	9,47	50,43	6,83
<i>C. sphaerospermum</i>	2,03	5,98	0,49

Table 4. Occurrence (%) of the main *Aspergillus* species

		HS			DG18		
	t° of incubation	Room n = 739	Outside n = 117	Diffuser n = 205	Room n = 739	Outside n = 117	Diffuser n = 205
<i>Aspergillus spp.</i>	25°C	14,21	41,88	9,76	18,00	50,43	15,12
<i>A. flavus 25</i>	25°C	0,27	2,56	0,00	0,00	0,00	0,00
<i>A. fumigatus</i>	25°C	5,41	21,37	4,88	2,30	7,69	2,44
<i>A. fumigatus</i>	45°C	7,31	41,03	4,39	/	/	/
<i>A. glaucus gr.</i>	25°C	0,68	4,27	0,98	7,31	32,48	7,32
<i>A. nidulans</i>	25°C	0,27	2,56	0,00	0,00	0,00	0,00
<i>A. niger</i>	25°C	2,30	8,55	1,46	2,98	5,13	2,93
<i>A. ochraceus</i>	25°C	0,68	0,86	0,49	0,68	0,00	0,00
<i>A. penicillioides</i>	25°C	0,00	0,86	0,00	0,00	0,00	0,00
<i>A. restrictus</i>	25°C	0,00	0,86	0,00	0,14	0,85	0,00
<i>A. ustus</i>	25°C	0,27	0,00	0,00	0,27	0,00	0,00
<i>A. versicolor</i>	25°C	1,62	1,71	0,49	1,35	4,27	0,00

Group 3 (Total *Penicillium*): the genus *Penicillium* contains many ubiquitous saprophyte species, whose conidia are easily distributed in the air. This characteristic may explain higher occurrence outdoors (with 64 to 75%) than indoors (with only 24 to 26% in room air, and 16 to 19% at diffuser air (Table 5)). Nevertheless, the maximum concentrations obtained in indoors conditions (630 cfu/m³) were considerably higher than the maximum results obtained in outdoor air (225 cfu/m³) (Table 5). These results confirm an indoor origin of *Penicillium* when present in high levels, for the same reasons as *Aspergillus*.

Group 4 (Total of other taxa): the total of other colonies were counted and examined for the toxigenic species (group 5), which generally appear only in low concentrations with this method.

Group 5 (Specific contaminant(s)): if one or several specific and dominant species are present in high quantities on strips, a specific evaluation and identification to species level is necessary. This is true for all fungi, even for the 3 first groups *Cladosporium*, *Aspergillus*, and *Penicillium*. In this study, percentage level 95 could be considered as an alert value. In this kind of environment (outdoor air filtered), it was rather low, 50 cfu/m³ maximum whatever the mold species, both in room or at diffuser air, and both on HS or DG18 media. So, each taxa above 50 cfu/m³ (4 cfu/strip at 25°C for 80 liters of sampled air) should be considered as a possible specific contaminant which requires a more detailed identification. Other colonies were not identified and placed in the group 4.

Table 5: Airborne molds concentration in cfu/m³, for the 3 genera present in the highest occurrence

TAXA	ORIGIN	MEDIA	N _x	P5 ¹	P25	P50	P75	P95	MAX
<i>Total Cladosporium</i>	Outdoor	HS	59	<13	<13	25	113	<u>400</u>	<u>788</u>
		DG18	38	<13	<13	13	25	<u>138</u>	<u>275</u>
<i>Total Cladosporium</i>	Diffuser	HS	195	<13	<13	<13	13	50	133
		DG18	189	<13	<13	<13	<13	38	<u>88</u>
<i>Total Cladosporium</i>	Room	HS	684	<13	<13	<13	13	50	<u>688</u>
		DG18	677	<13	<13	<13	13	38	<u>213</u>
<i>Total Aspergillus</i>	Outdoor	HS	73	<13	<13	<13	<13	13	113
		DG18	66	<13	<13	<13	25	38	63
<i>Total Aspergillus</i>	Diffuser	HS	201	<13	<13	<13	<13	<u>13</u>	<u>2225</u>
		DG18	202	<13	<13	<13	<13	<u>13</u>	<u>1888</u>
<i>Total Aspergillus</i>	Room	HS	707	<13	<13	<13	<1	13	<u>2225</u>
		DG18	712	<13	<13	<13	<13	13	<u>2038</u>
<i>Total Penicillium</i>	Outdoor	HS	71	<13	<13	13	38	75	175
		DG18	57	<13	<13	13	38	113	225
<i>Total Penicillium</i>	Diffuser	HS	201	<13	<13	<13	<13	25	<u>650</u>
		DG18	202	<13	<13	<13	<13	25	<u>238</u>
<i>Total Penicillium</i>	Room	HS	708	<13	<13	<13	<13	38	<u>650</u>
		DG18	705	<13	<13	<13	<13	38	<u>475</u>

¹ P = percentile

Group 6 (Specific toxigenic species in low concentration): very toxigenic species which may be under-estimated for different reasons were classified in this group. Presently only *Stachybotrys chartarum* (syn. *S. atra*) was listed. It is a strong hygrophilic species, and its spores are quickly desiccated in the air (Hintikka, 1998). So, even in case of large contamination, a great proportion of airborne spores may be not viable. Moreover, DG18 and any other osmophilic media are not adapted for this species (Chasseur, 1994). This group should be updated in the future, with new toxigenic species.

CONCLUSION

In a total fungal evaluation in three strategic areas, we found a simple path for a microbial evaluation of the building. This information may be useful for the technician for a good maintenance process, but also to evaluate the effectiveness of cleaning and disinfection. But, it is important to add that these quality levels were not directly correlated with health risks. At this stage, if an abnormal situation appears, a more detailed analysis is necessary with some molds species identifications.

ACKNOWLEDGEMENTS

We thank A. M. Verhaegen, V. Michel, and D. Philips for technical help, and G. Oebel (AIB-Vinçotte) who realised some of these surveys.

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MICROBIAL INDOOR AIR QUALITY IN OFFICE BUILDINGS WITH CENTRAL AIR CONDITIONING INSTALLATIONS IN BELGIUM. AN EASY TOOL FOR A BACTERIAL EVALUATION

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ABSTRACT

From 1996 to 1999, 166 surveys were carried out in office buildings equipped with central heating, ventilation and air-conditioning (HVAC) systems. Mesophilic environmental bacteria (EnvB, 25°C), mesophilic human-source bacteria (H-SB, 37°C) and thermophilic bacteria (ThB, 52°C) were collected in offices, at the diffuser outlet and in room air at breathing level, and outdoors in front of the air intake. Presence of ThB in offices air is rare, despite their presence in the humidifiers. Centiles 5, 25, 50, 75 and 95 were calculated for different bacterial groups to serve as a baseline for interpretation of results which are presented on a special graph to express the possible bacterial concentrations imbalances.

INDEX TERMS: Airborne Bacteria, indoor air, workplaces with HVAC systems, graphic representation

INTRODUCTION

Sick Building Syndrome (SBS) complaints (Apter, 1994) or Building Related Illness (BRI) (Seltzer, 1994) may be linked to various microbiological contaminations (Husman, 2000). Most bacteria do not pose a risk for human health. Nevertheless, some may generate illness or other complaints in indoor environment, especially if they thrive in appropriate building conditions, or when they are generated in very highly contaminated outdoor sources. *Legionella pneumophila* is presently the most dangerous infectious human bacteria which, under specific con-

ditions, is also able to grow in buildings or dwellings (Plouffe, 1999). But although bacteria are best known as agents of infectious disease, in the case of SBS or BRI, our attention is more focused on their allergenic or toxic effects. Some non-pathogenic bacteria developing on building material have been proved potentially harmful agents. Many studies have now shown a relationship between exposures to dust containing bacterial endotoxins, and acute as well as chronic disease symptoms in humans (Rylander, 1989; Michel, 1999; Gereda, 2000). Other toxic substances are also produced by some indoor bacteria, as *Bacillus cereus* isolated from settled dust and water damaged building materials (Anderson, 1998). Furthermore, toxic reactions and inflammatory cytokines in vitro produced by some strains of the genus *Mycobacterium* and of the species *Streptomyces griseus*, were demonstrated (Mikkola, 1999; Peltola, 1999). High exposure to gram-negative bacteria (GNB), by *Bacillus spp.*, or by some Actinomycetes as *Microspolyspora faeni* were reported in case of hypersensitivity disease (HP) (Otten, Burge, 1999).

In indoor air microbiology norms do not occur and *limit values* are rare (ACGIH, 1999; Chasseur, 2000). Moreover, the total indoor evaluation of bacteria does not give sufficient specific information about the microbial quality (kinds of bacteria) in the building. However, identifying all the isolated colonies is rarely useful. In an overview on indoor bacterial studies, Otten and Burge (ACGIH, 1999) reviewed the following basic distinction between mesophilic bacteria including Environmental Bacteria (EnvB, 18 to 30°C), Human-Source Bacteria (H-SB, 35 to 44°C), and thermophilic bacteria (ThB, 50 to 55°C) including *Thermoactinomyces*. Environmental mesophilic bacteria are generally coming from outdoors, but may also grow indoors, in water of humidifiers for instance. Human-source bacteria highly dominate indoor air in overcrowded environments or in poorly ventilated rooms. Thermophilic bacteria are rare, and their presence indicates an indoor contamination. So, on the basis of the incubation temperature, some important causes of an excessive level of indoor bacteria may be detected, due to a simple total evaluation of these 3 groups of bacteria. Constructed with percentiles, a quality scale should be an easy tool to compare results with those from other buildings, but it does not constitute any help for evaluation of health risks.

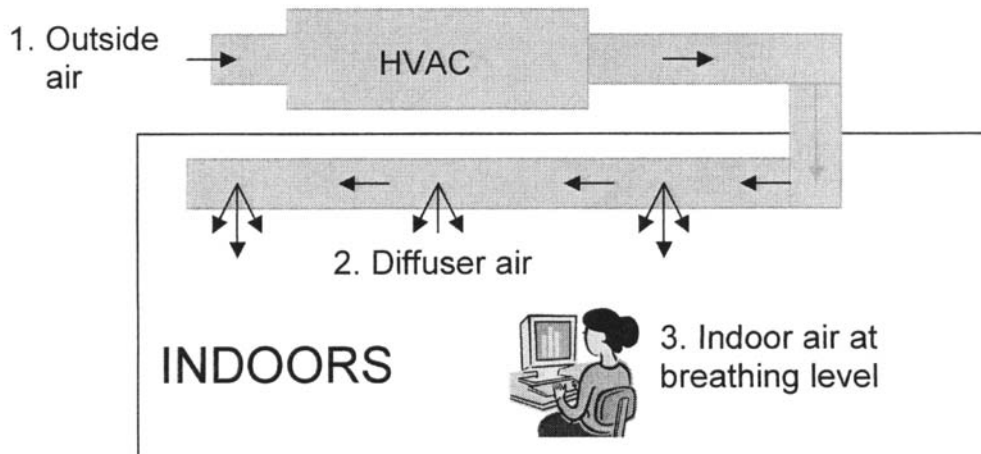
METHODS

From 1996 to 1999, 166 surveys were realized in office buildings equipped with a central HVAC system. A total of 1110 indoor air samples were used for different data analyses. Mesophilic environmental bacteria (EnvB, 25°C), mesophilic human-source bacteria (H-SB, 37°C) and thermophilic bacteria (ThB, 52°C) were evaluated in the offices. For each sample, a volume of air was taken with the Reuter Centrifugal Sampler (RCS+) from Biotest (Kaye, 1988; Ljungqvist, 1990, 1993; Mehta, 2000), 80 liters for mesophilic bacteria, and 160 liters for thermophilic bac-

teria. Three strips filled with TSA medium were used per location, one incubated for 5 days at 25°C (EnvB), the second for 2 days at 37°C (H-SB), and the last incubated for 2 days in a humid atmosphere at 52°C (ThB).

Outside air sampling was also carried out as reference during surveys, in front of the outside inlet duct (126 samples). In the offices, the air was sampled in *the breathing zone* (812 samples), and at the diffuser outlets (223 samples).

Figure 1. Three strategic places for air sampling



Statistic treatment for outdoor mesophilic bacteria: The aim of the time series analysis was to find a general trend over the four years investigated and to detect possible seasonal and thus month effects. An additive model was chosen. A linear regression procedure was used to detect a global trend. In order to further detect possible differences between time effects, a larger regression model was used which included each month component and the trend as independent variables. Each month effect was compared to a baseline month component which was chosen as the closest to the average level of the 12 month components. The Trends procedures included in the SPSS10.0 statistical software were used.

RESULTS AND DISCUSSION

Outdoor air: seasonal variations: October was chosen as a baseline month for EnvB species. The analysis of the monthly components indicated three months with a significant higher concentration: January ($p=0.043$), September ($p= 0.010$), and May ($p= 0.005$). For H-SB species, April was chosen as the baseline month, and again three months presented a higher concentration: June ($p=0.025$), July ($p=$

0.002) and September ($p=0.006$). But these months are not part of a determined and full season.

In a second step, the difference (Δ) between the building indoor data average and each corresponding outdoor concentrations was calculated, with April as baseline month for EnvB species. The analysis of the monthly components indicated only one month with a higher concentration, January ($p=0.030$), and only one month with a weaker concentration, March ($p=0.020$). For H-SB species, February was chosen as the baseline month: three months presented a significant higher concentration: January ($p=0.012$), October ($p=0.033$), and November ($p=0.15$). In this case, also two months presented a weaker level: September ($p=0.001$) and June ($p=0.012$). Some significative monthly variations were thus observed but no determined seasonal trend appeared.

Outdoor air: limit values: Outdoor bacteria concentrations being not or weakly subject to seasonal variations, percentiles may be calculated. On table 3, percentile 50 (median) was 213 cfu/m³ for EnvB, and 63 cfu/m³ for H-SB. But maximum values reached 2738 cfu/m³ for EnvB, and 1525 cfu/m³ for H-SB.

For thermophilic bacteria, essentially *Thermoactinomyces spp.*, the occurrence was weak with 9.76% for total *Thermoactinomyces*, 4.88% for *T. candidus* and 0.81% for *T. vulgaris*. So, for ThB, no statistic treatment was necessary in order to prove a possible seasonal variation. Moreover, the maximal airborne concentration was only 13 cfu/m³ on a total of 117 samples.

Indoor air: The general trend for all the examined buildings, was an increase of H-SB from outdoors (Percentile (P) 50=63) to indoors (P50=113), especially for the 5% most contaminated buildings where 2625 (EnvB) and 5750 (H-SB) cfu/m³ were recorded. On the contrary, the EnvB were more numerous outdoors (P50=213) than indoors (P50=125), but this difference was narrower for the 5% most contaminated buildings, which in these cases, may suppose important indoor developments. In front of the diffusers in offices, H-SB levels (P50=75) remained lower than EnvB levels (P50=100), but this difference was weak. The difference between H-SB (P50=125) and EnvB (P50=150) in room air, indicated the same trend. These observations may be explained as well by the percentage of bad air recycling for economy of energy (increase of indoor H-SB) than by filtration of outdoor air (diminution of indoor EnvB species). It may be assumed that high levels of EnvB at diffusers, as recorded maxima of 1126 cfu/m³, were due to bacterial development in the HVAC system, and that high levels of H-SB at diffusers, as recorded maxima of 913 cfu/m³, could be due to too much recycling indoor air (Table 1). Thermophilic bacteria were rarely present in indoor air of this environment, with an occurrence of only 2.93% at the diffuser outlets, and 2.30 % in room air (Table 2). The maximal concentration obtained in ambient air was 25 cfu/m³.

However, presence of *Thermoactinomyces* was sometimes detected, especially near or on warm coils. These results showed that other more relevant places should also be examined, using different methods, as RODAC plates for instance.

Table 1. Percentiles expressed in cfu/m³ for airborne bacteria calculated with results obtained during 166 microbiological surveys in buildings equipped with a HVAC system.

Percentile	Outdoors		Indoor Total		Indoor Room air		Indoor diffuser	
	EB, 25°C	HSB, 37°C	EB, 25°C	HSB, 37°C	EB, 25°C	HSB, 37°C	EB, 25°C	HSB, 37°C
5	50	0	13	0	25	13	0	0
25	100	25	63	50	75	63	38	25
50	213	63	125	113	150	125	100	75
75	363	138	250	200	275	238	175	138
95	713	450	563	500	588	550	375	338
n	122	126	1107	1110	809	812	223	223
Max.	2738	1525	2625	5750	2525	1850	1126	913

EnvB: Mesophilic Environmental Bacteria;
H-SB: Mesophilic Human-Source Bacteria

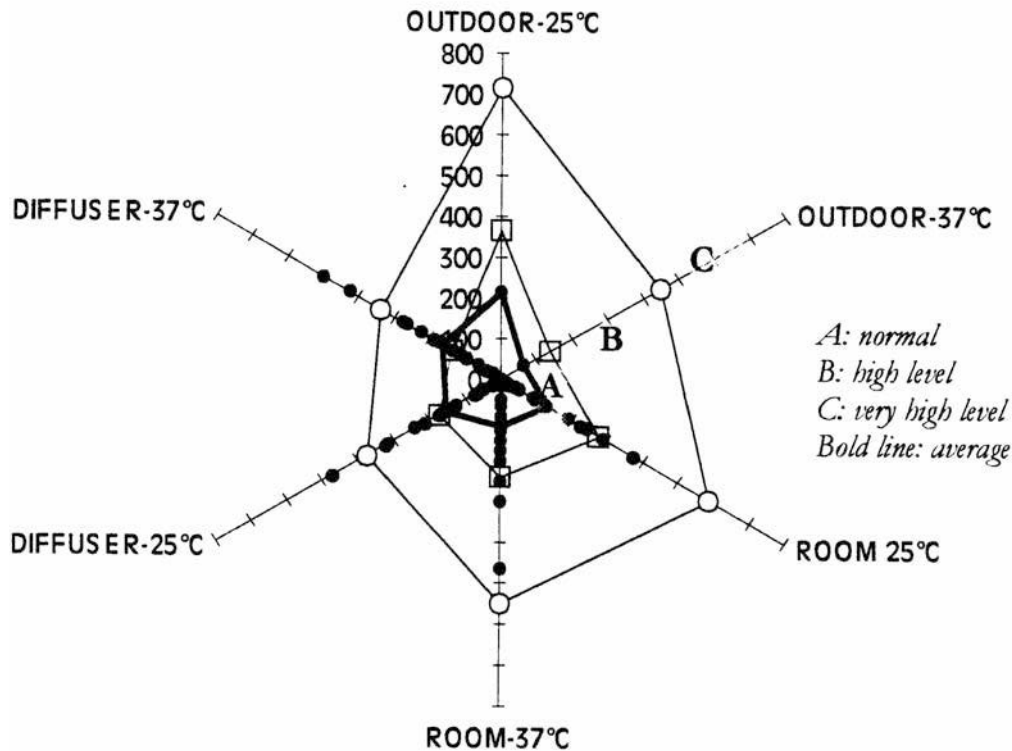
Table 2. Occurrence (%) of airborne *Thermoactinomyces* (ThB) in 166 surveys in buildings equipped with HVAC

	Total	<i>T. vulgaris</i>	<i>T. candidus</i>
Outdoors	9,76	0,81	4,88
Diffuser	2,93	0,00	1,46
Room air	2,30	0,00	1,08

Graphic representation of results: To facilitate data interpretation, a graphic representation was plotted. Three bacterial quality levels were delimited in the background, by using different percentiles results to serve as a baseline for interpretation: normal (below percentile 75), high (between percentile 75 and 95) and

very high (above percentile 95). Moreover, results were reported on 6 axes, corresponding to bacteria H-SB and EnvB sampled in 3 strategic system areas: outdoors during the survey and in workplaces (ambient and diffuser air). In HVAC, outside air is filtered, contrary to an open environment such as a dwelling.

Figure 1. An example of graphic representation of microbial survey in building with HVAC



It is important to note that these numerical levels for various categories of bacteria were not correlated with health risks. They can and should be used as a tool to compare results with those from other buildings. With this graphic representation, a global picture of the airborne bacterial quality was obtained, allowing to visualize trends and imbalances, and to inform about a possible contamination.

This approach is particularly useful to the technician for a good maintenance process or to evaluate the effectiveness of cleaning and disinfection.

An example of survey is commented (Figure 1). In this case, results were in the high level range and some reached very high levels. The outdoor air was normal. But after circulating in the air conditioning installation, the airborne bacterial charge increased, which was visualized on the graph by an imbalance at this level. We could conclude that the mechanical ventilation system should be examined. On

the other hand, mechanically extracted air seemed sufficient because room air remained rather less contaminated than diffuser air.

CONCLUSION

Two categories of bacteria in three strategic areas were measured. In this case, bacteria concentrations are rather an indicator of the problem. Second, places with microbial amplification (humidifier) are generally detected by high levels of EnvB in air, with all the potential risks for health due to endotoxins or MVOC, which should be implicated in pathologies that cannot be diagnosed such as SBS.

ACKNOWLEDGEMENTS

We thank A. M. Verhaegen, V. Michel, and D. Philips for technical help, and G. Oebel (AIB-Vinçotte Ecosafer) who made some of these surveys.

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COMPARISON OF PVC CASSETTES VERSUS VACUUM BAGS FOR COLLECTION & ENUMERATION OF CULTURABLE FUNGI IN SETTLED DUST

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ABSTRACT

A comparison of two collection techniques for measuring settled dust in water damaged single-family homes, multi-family homes, public buildings, and schools was undertaken. One technique utilizes PVC or MCE cassettes and a portable vacuum pump, the other uses a vacuum cleaner to capture dust in DuPont HySurf™ allergen filter bags. Preliminary results indicate poor correlation between the two different types of settled dust sampling techniques in terms of numbers of spores recovered per gram of collected settled dust. Species distributions as measured by rank order analysis however appear to be better correlated.

INTRODUCTION

Over time, migration of spores inward from wall cavities with water damage will result in the accumulation of transported spores into soft furnishings, carpeting, and other settled dust reservoirs of a building (Cole 1996, Roberts 1999). Normal vacuuming may not remove accumulated particulate material in carpeting (Roberts 1996).

For other particulate material which accumulates in carpeting, such as lead, loadings in carpeting and on surfaces has been shown in some studies to be related to body burden (Roberts 1999). Carpet dust has been related to complaints of sick building syndrome in the Danish Town Hall Study (Gyntelberg 1994) and in studies of chemical pollutants (Camann 1994).

Previous studies have suggested the use of air samples, deposited dust samples analyzed by culture methods, and/or spore counting to assess risk to occupants of mold spore exposure (Cole *et al.* 1996, Dillon *et al.* 1996). Swab sampling of surfaces followed by culture or direct microscopic examination has also been used by some practitioners as a characterization method.

Because short-duration air samples represent momentary conditions, settled dust samples may represent longer-term exposure (Dales *et al.* 1997).

Care must be taken to avoid areas such as carpeting which themselves have become wet and have been supporting mold growth. Inclusion of such areas would positively bias the results. Such a sample would not reflect historical air levels or characterize a particular reservoir, but rather indicate immediate local conditions.

Microbiologic analysis of sieved (<250 microns) settled dust may be through serial dilution and plating on agar media (Dillon *et al* 1999). There may be significant differences in the species distribution obtained between the two methods (Miller, 2001; Dillon *et al* 1999). No standard laboratory method exists for this analysis. Some laboratories request a minimum quantity of dust and utilize alternative analysis methodology when the quantity of recovered dust is below the required amount. Processing variables in the laboratory can affect results; a laboratory protocol for analysis of collected dusts has been proposed (Macher, 2001).

Reporting is typically in Colony Forming Units (CFU) per gram of sieved collected dust. Interpretation may be unrelated to quantitative CFU/m³ data, but more dependent on a rank order analysis by species as used for air samples in the AIHA Field Guide (Dillon *et al* 1999, p-52).

Direct microscopic examination of the dust has also been used for analysis (Morey 1994). This technique is limited in its ability to distinguish between certain visually similar organisms.

METHODS

The vacuum method utilizing allergen collection bags with a high-efficiency (97% efficient at 1.1micron particle size) DuPont HySurf™ wet-laid polyethylene filter media (supplied by Midwest Filtration, Fairfield, OH 513-874-6510). Vacuuming was conducted with a Euroclean UZ930 vacuum (approximately 80 CFM at 90” W. C.) with HEPA-filtered exhaust. At least 1-3 square meters was vacuumed. The bag was inserted directly into the vacuum hose without a collection nozzle.

The cassette (mini-vacuum) method utilized a PVC filter cassettes (pore size 0.8 microns) or mixed cellulose ester (MCE) filter (pore size 0.8 microns) operated at 15 LPM open-faced. At least 1-3 square meters was vacuumed.

Settled dust was collected sequentially from each room or rooms using both techniques. In both techniques, the investigator was required to crawl on the floor in order to properly apply the collection device to the carpeting. High traffic areas, areas under plants, and areas adjacent to walls suspected of moisture incursion were avoided so as to minimize bias samples with dirt tracked in on shoes or by

collecting spores from colonized materials. Samples were shipped without refrigeration.

For both techniques, the dust was sieved by the laboratory prior to weighing and plating. Analysis was by dilution plating on DG-18 and Malt Extract agar media with the Laboratory requesting a minimum of 100 mg. of sieved dust per sample. When sample loading was too low to recover sufficient sieved dust for analysis, the cassette was washed and the resulting effluent plated as described above. Reporting was by colonies recovered for each individual species present, with a total CFU/g result for each identified species and a CFU/g sum for all recovered species. For DG-18 and Corn Meal Agar plates, *Penicillium* and *Cladosporium* were reported to e. c. level; for the Malt Extract Agar plates, *Penicillium* was reported by e. c. and species.

68 pairs of data (136 total dust samples) were obtained from 14 different buildings. Each sample was plated on three media, yielding a total of 414 culture plates analyzed. These buildings comprised single family homes, apartment buildings, public buildings, schools, and condominiums. All were suspected of water leakage into the building envelope due to construction defects. No control buildings were evaluated. In most cases, samples were taken from single rooms. In some cases, the samples were composited across one or more rooms.

RESULTS

Quantities of dust recovered via the two techniques varied, with the vacuum bag technique recovering more dust in a shorter period of time. In some cases when the environment was very clean, it would have been difficult to collect a similar quantity of dust using the cassette technique. The minimum quantity of 100 mg. of dust plated was more easily obtained when the sample was obtained by allergen bag (91%, 62/68) than by cassette (43%, 29/68). The average quantity of dust plated was higher using the allergen bag technique than the cassette technique (0.13028 grams versus 0.08004 grams). Some of the cassette samples had very low quantities of dust recovered (6 samples recovered less than 0.02 grams of sieved dust; none of the bag samples recovered less than 0.02 grams).

The average total colony-forming units recovered using the two collection techniques differed. For each sampling media (Malt Extract agar, DG-18 and Corn Meal Agar) between 52 and 55 of the 68 paired samples indicated higher CFU/gram levels for the cassette samples versus the vacuum bag samples. There was consistency across the plating media for the average total CFU/gram of spores recovered by dilution plating (Corn Meal Agar, approximate average CFU/gram for cassette method 716,000, for vacuum bag method 207,000; DG-18 Agar, average CFU/gram for cassette method 788,000 for vacuum bag method 244,000; Malt

Extract Agar, average CFU/gram for cassette method 706,000, for vacuum bag method 233,000).

Of the total samples collected by the cassette (mini-vacuum) method, independent of which media was utilized, 79% had higher CFU/gram values compared with those collected via allergen collection bags (161 of 204 plate pairs, $p < 0.01$).

When rank order analysis was conducted, agreement between the two techniques on the most predominant recovered organism occurred 34/65 times for the corn meal agar, 35/65 times for the DG-18 agar, and 36/64 times for the malt extract agar. Between 10 and 15 additional pairs for each type of agar either had the rank orders reversed for the first and second rank, or had one taxon common between the two techniques for the first and second most commonly recovered organism. Thus it appeared that 55% to 75% of the time, there was agreement between the two collection techniques if rank order interpretation were utilized.

In buildings with water damage, the commonly recovered species included filamentous soil fungi known to grow on wet building materials. The most commonly recovered organisms are shown in Table 1. *Penicillium* species, including *P. aurantiogriseum*, yeasts, and *Cladosporium sp.* were the three most commonly recovered organisms using both techniques.

Table 1. Frequency of recovery (first or second rank order) of various fungi from settled dust utilizing three culture media (total = 414 plates)

	CMA	DG-18	MEA
<i>Penicillium</i> species	94	100	97
<i>Rhodotorula sp.</i>	18	13	17
<i>Cladosporium</i> species	26	46	33
<i>Phoma sp.</i>	n.d.	5	5
<i>A. versicolor</i>	24	26	27
<i>A. ustus</i>	2	2	1
<i>A. sydowii</i>	2	2	2
<i>S. chartarum</i>	4	1	1

Table 2 lists the recovered organisms by frequency for both sampling media and by plate type.

Both collection techniques appeared equally sensitive in permitting the growth of numerous filamentous molds such that no difference in interpretation was evident

if the interpretation criteria were based on the recovery of a particular organism rather than a rank order analysis.

Table 2. Frequency (percent) of recovery (first or second rank order) of selected organisms for cassette and vacuum bag by plating media

		CMA	DG-18	MEA
<i>Penicillium</i> species (including <i>P.aur</i>)	Cassette (n = 68)	50	56	55
	Bag (n = 68)	44	44	42
Yeast	Cassette (n = 68)	22	17	13
	Bag (n = 68)	35	22	31
<i>Rhodotorula sp.</i>	Cassette (n = 68)	5	1	4
	Bag (n = 68)	13	12	13
<i>Cladosporium species</i>	Cassette (n = 68)	14	25	18
	Bag (n = 68)	12	21	15
<i>P. aurantiogreiseum</i>	Cassette (n = 68)	-	-	22
	Bag (n = 68)	-	-	17
<i>P. variotti</i>	Cassette (n = 68)	2	1	3
	Bag (n = 68)	1	nd	nd
<i>A. vericolor</i>	Cassette (n = 68)	13	15	15
	Bag (n = 68)	11	13	12
<i>A. sydowii</i>	Cassette (n = 68)	1	2	1
	Bag (n = 68)	1	nd	nd
<i>S. chartarum</i>	Cassette (n = 68)	1	nd	nd
	Bag (n = 68)	3	1	1

DISCUSSION

It is unclear why numerical differences in total recovered fungal colonies exist between the two techniques.

In most cases, the numbers of serial dilutions reported from one technique differed from the other. A magnitude difference could possibly be explained by a multiplication effect in the calculations required by the serial dilution process. This effect may be confounded by the total quantity of dust recovered, which differed via the two techniques.

Differences in viability may exist between the two techniques. The higher vacuum associated with the allergen bag collection technique may capture a greater volume

of dust, especially dust located deeper in the carpeting, but may possibly be associated with desiccation of the spores, reducing viability on culture. The allergen bag may be torn during the sampling process resulting in selective by-pass of small mold spores. The lower collection efficiency of the bag may also be a factor. The cassette method may selectively capture lighter, smaller particulate, reducing the contribution of non-fungal particulate, e.g. heavy mineral dusts to the denominator. No evidence to evaluate any of these theories currently exists.

The allergen bag technique may permit multiple analyses from a larger quantity of dust recovered, facilitating additional analyses and/or archiving of samples. The cassette is less expensive than the allergen bag, though the per sample difference is only a few \$U.S. Cassettes are readily available to industrial hygienists, whereas allergen bags are not used extensively. Portable sampling pumps, used in the cassette technique, may be readily available due to coincident air sampling whereas a portable vacuum cleaner, used for allergen bag sampling, may not be as readily available.

The quantities of specific organisms collected differed between the two techniques for *Rhodotorula* and yeasts, and possibly with *S. chartarum*, with higher recoveries from the allergen bag versus the cassette. This observation may be due to chance due to the low numbers of samples in this study.

Epidemiologic studies which include information on mold spores as well as other biologically active particulate have used allergen bags more frequently than cassettes. It may therefore be easier to apply dose/response information derived from these studies to data collected in a similar manner.

This study did not identify a preference for either technique. If rank order analysis or simple presence of an organism is the interpretive criteria to be applied to settled dust samples, then the differences between the two techniques may be of limited significance. If numerical interpretation were conducted (not recommended), results from one technique appear to be incomparable with the other technique, due to the differences noted in this preliminary study.

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ASSESSMENT OF MOLD SOURCES IN INDOOR ENVIRONMENTS

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ABSTRACT

The airborne fungal spore concentration measured with bioaerosol samplers during specific time intervals may not be representative of indoor air quality because of sporadic nature of the spore release. An alternative to the air sampling would be an assessment of the spore release from the mold-contaminated indoor sources under the most favorable aerosolization condition. We have recently developed a new Fungal Spore Source Strength Tester (FSSST) for assessing the potential of the aerosolization of fungal spores from moldy surfaces. The experiments revealed that the spores were efficiently aerosolized at a rate ranging from 10^2 to 10^3 spores per cm^2 per min (total for all genera). The aerosolization efficiency was determined for different fungal genera and building materials. The data suggest that the FSSST provides the most favorable conditions for the spore aerosolization and thus can be used for the assessment of the maximum potential release from a fungal source.

INDEX TERMS: Bioaerosols; sampling; spore aerosolization; aerosol concentration; source strength.

INTRODUCTION

Health effects on both children and adults have been reported to increase when visible fungal growth is detected on indoor surfaces (Miller 1995; Verhoeff *et al.* 1995; Hu *et al.* 1997; Siggaard *et al.* 1999). The airborne fungal spore concentration measured with bioaerosol samplers during specific time intervals may not be representative of indoor air quality. In many situations, indoor air sampling has not been sufficient in identifying mold problems in housing or establishing a relationship between the airborne mold concentration and health effects. This can be explained by a sporadic nature of the spore aerosolization, i.e., the mold sporulation might not necessarily have occurred during the air sampling period. An alter-

native to the air sampling would be an assessment of the spore release from the mold-contaminated indoor sources under the most favorable aerosolization conditions (the “worst case scenario”). The spore release from growth surfaces is driven by the energy from external sources and may be significantly affected by environmental factors. The aerosolization caused by air currents seems to be the most prevalent mechanism for indoor fungi (Gregory 1973; Madelin 1994). The amount of fungal spores released from agar (Pasanen *et al.* 1991) and fiberglass duct material (Foarde *et al.* 1999) was found to increase with increasing air velocity and decrease with increasing air humidity. Kildesø *et al.* (2003) studied the spore release from wet building materials and found that it is very different for different fungi under identical conditions. In order to characterize the “worst-case” scenario of the air mold contamination from identified indoor sources, it is important to assess these sources under the conditions, which are most favorable for aerosolization. The Fungal Spore Source Strength Tester (FSSST) was used in this study to assess the mold source and its aerosolization potential in indoor environments with different building materials.

MATERIALS AND METHODS

The Fungal Spore Source Strength Tester (FSSST, Grinshpun *et al.* 2002) is a cup-like device held against the test surface. The spores are aerosolized by air jets oriented towards the surface. The device utilizes two vacuum pumps (push and pull). The spores are released from the growth surface by HEPA-filtered air (1244 HEPA capsule filter, PALL Gelman Laboratory, Ann Arbor, MI). The air jets are generated by a pump upstream of the cup. The released spores are collected using a BioSampler (SKC Inc., Eighty Four, PA). The samples are subsequently enumerated by microscopic methods.

Four residential homes with visible mold-contaminated surfaces including a concrete surface, painted dry wall, particleboard and wooden logs were selected in the Greater Cincinnati area. The tester was applied to the contaminated surfaces for 5 to 20 minutes at an airflow rate of 11.5 L/min and the aerosolized spores were collected into the BioSampler connected to the FSSST outlet at 12.5 L/min. The slight difference in these flow rates allowed preventing the air contamination in the vicinity of the FSSST. Simultaneously, indoor air sampling was performed with the BioSampler to measure the airborne concentration of fungal spores in the tested environment. Prior to each experiment, surface samples were taken from the mold-contaminated areas by using a sterile swab (Becton Dickinson & Co., Sparks, MD) to determine the spore surface density in the source. The surface samples were extracted into 20 mL of sterile water by 10 min vortexing with Vortex touch mixer (Model 231, Fisher Scientific Company, Pittsburgh, PA). The liquid samples from the BioSampler and the surface swab samples were filtered onto 13-mm mixed cel-

lulose ester filters (0.8 μm pore size) for microscopic analysis. The filters were placed on glass slides and cleared by acetone vapor using a modified instant acetone-vaporizing unit (model: Quickfix, Environmental Monitoring Systems, Charleston, SC). The resulting sample was mounted on the slide using glycerin jelly with a square 25 x 25 mm cover glass. The samples were enumerated with respect to the total spore concentrations as well as the individual spore types.

RESULTS AND DISCUSSION

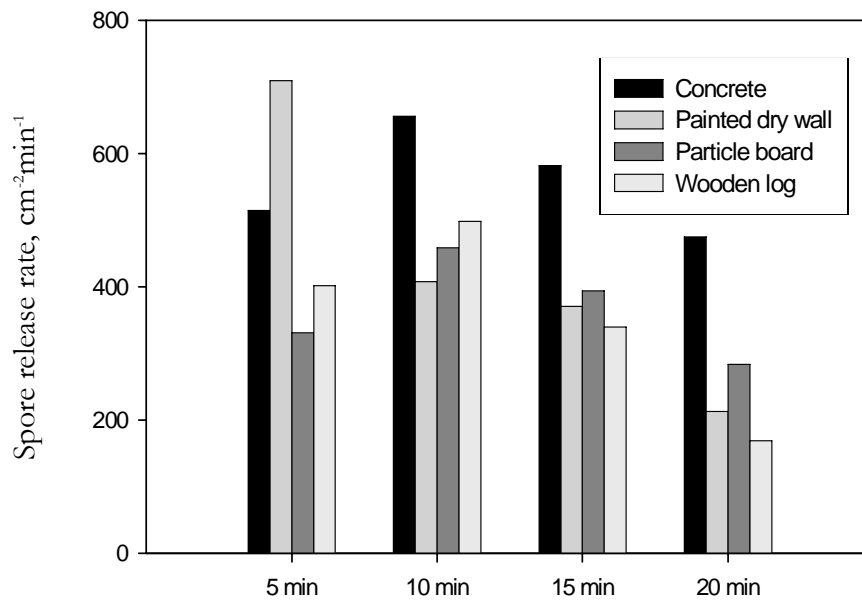
The experiments revealed that the spores were efficiently aerosolized from the contaminated surfaces by the FSSST. Figure 1 shows the total spore release rate for different contaminated surfaces as measured during 5, 10, 15, and 20 min tests. Based on the averaging the data obtained at these time intervals, the rate was 557 ± 79 spores $\text{cm}^{-2} \text{min}^{-1}$ for concrete surface, 425 ± 208 spores $\text{cm}^{-2} \text{min}^{-1}$ for painted dry wall, 367 ± 76 spores $\text{cm}^{-2} \text{min}^{-1}$ for particleboard, and 353 ± 139 spores $\text{cm}^{-2} \text{min}^{-1}$ for wooden log. The spore release rate was greater during the first 10 min than during the last 10 min of the 20-minute time interval of the FSSST application. This finding confirms the results of our earlier laboratory study (Sivasubramani *et al.* 2002) performed with artificially contaminated (inoculated) building materials. It was concluded that 10-minute application of the FSSST is suitable for assessing the potential of the spore release from building materials.

The release of *Aspergillus/Penicillium* and *Cladosporium* from concrete surfaces was detected. *Aspergillus/Penicillium*, *Chaetomium*, *Cladosporium*, *Basidiospores* and *Stachybotrys* were found to be aerosolized from painted dry wall. The tests with particleboard revealed release of *Aspergillus/Penicillium* and *Chaetomium*. No genus other than *Cladosporium* was detected in the tests with wooden log.

The ratio of the number of spores aerosolized from 1 cm^2 of the moldy surface during the 10-min FSSST application to the spore surface density determined on the tested surface with the swab method was defined as the efficiency of spore release. Figure 2 shows this ratio (expressed in percent) for different contaminated surfaces. The efficiency of total spore release was $1.56 \pm 0.22\%$ for concrete surface, 0.62 ± 0.30 for painted dry wall, $0.03 \pm 0.01\%$ for particleboard, and $0.02 \pm 0.01\%$ for wooden log. The efficiency of *Aspergillus/Penicillium* spore release was lower for particleboard ($0.15 \pm 0.06\%$) than for painted dry wall ($2.30 \pm 1.22\%$) or for concrete surface ($1.48 \pm 0.17\%$). This may be due to the difference in temperature, relative humidity and surface moisture level of the contaminated surface. The efficiency of release of *Cladosporium* spores was higher for concrete surfaces ($1.68 \pm 0.32\%$) than for painted dry wall ($0.28 \pm 0.29\%$) and wooden log ($0.18 \pm 0.01\%$). Our result shows that the spore release rate was different for the same fun-

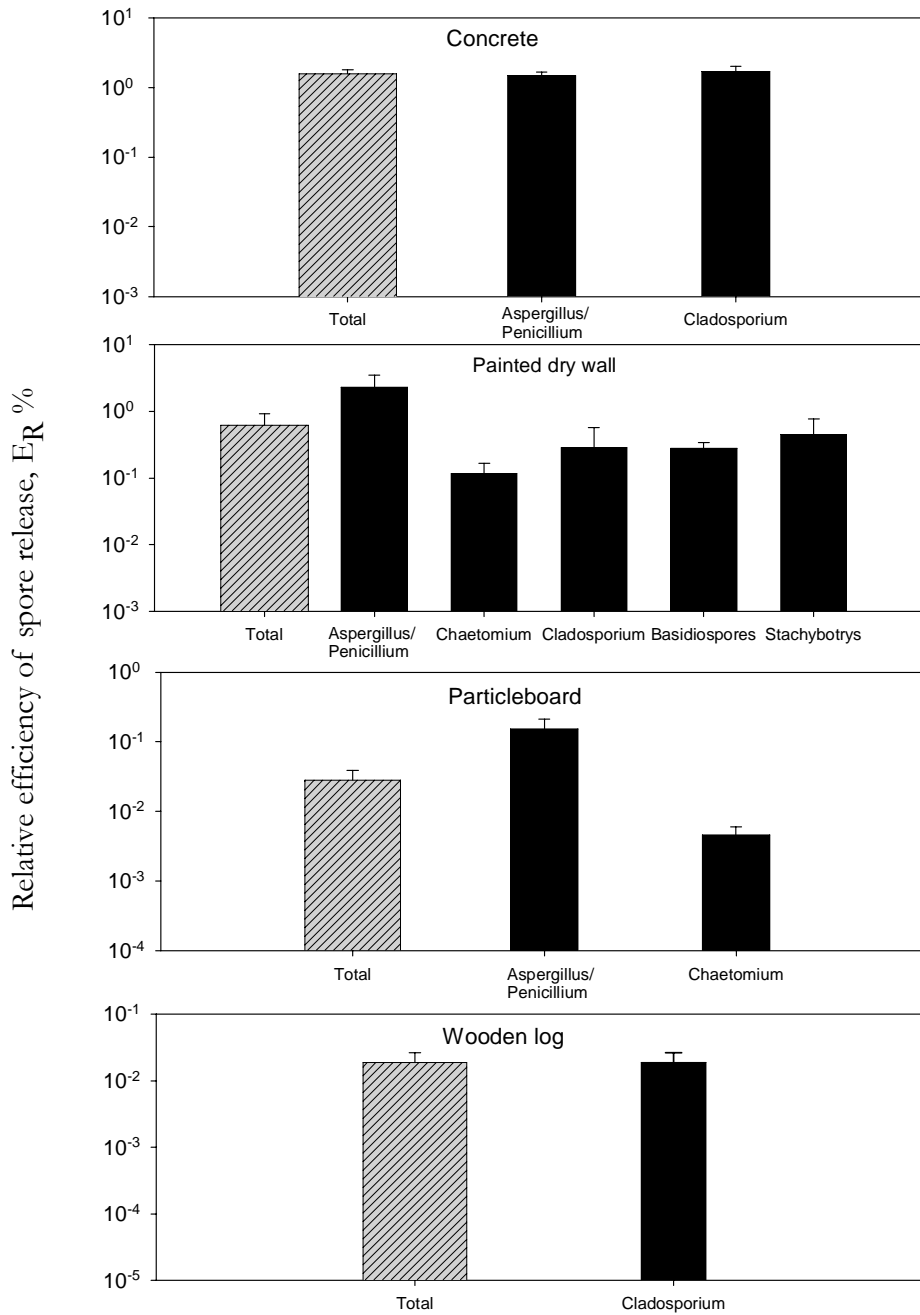
gal genera tested under different conditions, such as building material properties, surface moisture level, and the air temperature and humidity.

Figure 1. Spore release rate for different contaminated surfaces.



The experimental data on the spore release obtained in this study were utilized to estimate the resulting spore concentrations in the air. The concentration of airborne fungal spores in a room of $6 \text{ m} \times 5 \text{ m} \times 3 \text{ m} = 90 \text{ m}^3$ was estimated assuming that 1 m^2 ($\sim 1\%$ of the total surface area) was heavily contaminated with fungi and that the environmental conditions were most favorable for the spore release. The release rate was assumed to have the same value as the one measured with the FSSST during 10 minutes. We found that the spore concentration in the air ranged from 3×10^5 to $6 \times 10^5 \text{ m}^{-3}$. The measured concentration of airborne spores was in the range of $(1 - 17) \times 10^4 \text{ m}^{-3}$. Rao *et al.* (1996) reported that the typical total spore concentrations in water-damaged buildings were up to approximately 10^4 spores/ m^3 .

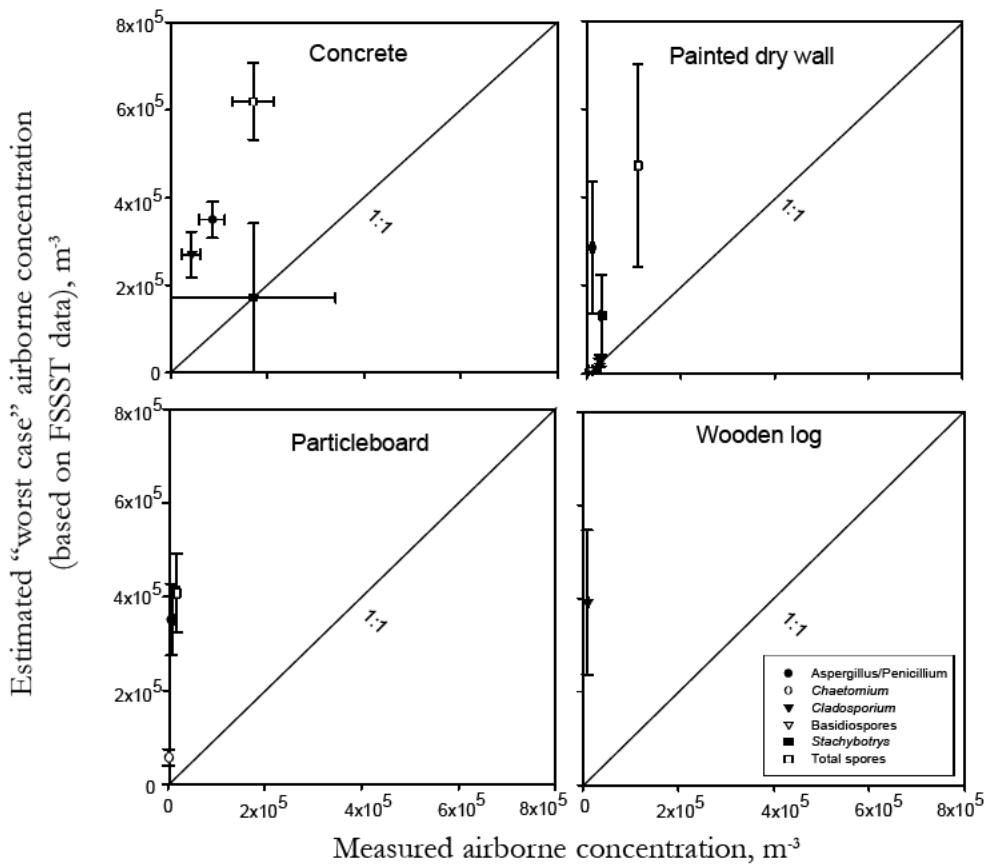
Figure 2. Efficiency of spore release during the 10 min FSSST application.



Error bars indicate standard deviation of four repeats.

The estimated airborne spore concentrations of different fungi are plotted in Figure 3 against the measured airborne spore concentrations. For all the four contaminated surfaces, the estimated spore concentration was greater than (or about the same as) the measured airborne concentration. This confirms the FSSST is suitable for assessing the potential of the spore aerosolization that may occur in the field when a variety of fungal species are released from growth surfaces to the air.

Figure 3. The estimated airborne spore concentration versus measured airborne spore concentration



FINDINGS AND CONCLUSIONS

The FSSST operating for 10 minutes provides very high spore release rates. This release produces the airborne spore concentration levels, which is much greater than the typical spore concentrations reported in water-damaged moldy buildings. It was concluded that the use of the FSSST allows assessing the maximum potential mold contamination of indoor air from an identified fungal source.

ACKNOWLEDGEMENT

The assessment of mold sources in indoor environments by using FSSST was supported by U.S. Department of Housing and Urban Development (Healthy Homes Research, Grant OHLHH0099-01). The authors appreciate this support. The authors are also thankful to Dainius Martuzevicius, Richard T. Niemeier, and Michael Yermakov for their assistance in the field sampling.

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Chapter 6
Assessment II – Field Findings (Continued) and
Mycology

Session chairs:

Anna-Liisa Pasanen, Claude Mainville

SAMPLING: A NEGLECTED PART OF BUILDING INVESTIGATIONS AND MICROBIAL EXPOSURE ASSESSMENT

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No detailed guidelines on the sampling strategies or recommended sampling methods for microbial analyses exist for building investigations or for the microbial exposure assessment. As regards building investigation, this is understandable, because every case is unique. The need of sampling and the design of the sampling strategy depend on the purpose of investigation: detection of contamination (site, extent and/or spread of contamination) or verification of the need and/or success of renovation. However, no conclusions on health risks can be made based on environmental microbial monitoring in individual cases, because there is no target or threshold limits for micro-organisms or their fragments.

In order to design the sampling strategy for the building investigation, appropriate sampling types (bulk material, surface, settled dust or air samples), sampling sites, periods and the number of samples of each type as well as appropriate sampling methods should be considered based on the background information and visual inspection of the building. Different sampling types serve different purposes and provide different information. Bulk material and surface samples are useful for detection of microbial contamination, while air sampling can be used to find out, whether microbes are spreading from contaminated areas to other parts of the building or whether renovation of the damage has succeeded (clearance sampling). A spatial and temporal variation in the microbial concentration inside the building affects the number of samples needed and consideration of appropriate sampling sites and times. A choice of the sampling method is a combination of the purpose of investigation, requirements of the analytical methods used for the sampling procedure (sample volume, sampling time, collection media) and reference data available for interpretation of the results. There is a large collection of different sampling methods with their advantages and defects, especially for air sampling (see Table 1).

There are differences in the microbial exposure assessment (sampling and analyses) between epidemiologic studies, which makes difficult to compare results of various studies. In general, too little attention has been paid to sampling strategies

in the research, whereas analytical methods have been as a target of scientific interest for years. However, even the most advanced, reliable and qualified analyses cannot correct errors made in sampling. Like in building investigations, the sampling strategy depends on the design of the field or epidemiologic study. However, models of recommended sampling strategies for different kinds of studies are needed. Requirements of the sampling strategy (e.g. the number of samples) in the scientific research are stricter than those of 'real life' building investigations e.g. because of demands for statistical analyses of the results. The microbial assessment relies on air or settled dust sampling and/or human samples and examination of exposed subjects. The preference for the air or settled dust sampling in the microbial assessment has been discussed actively, but no clear consensus has been achieved yet. As for the examination of exposed subjects, the validity of the methods and the collection of reference data are just at the beginning.

Though a large collection of air samplers is already available, new sampling methods for microbial analyses have been recently developed and the development is still in process. The most important properties for the air sampler are representativeness (sampling from the breathing zone and over exposure time, isokinetic sampling, and the high sampling efficiency for relevant particle sizes), reliability (precision, accuracy, and reproducibility), and practical aspects (easy to use). Though a possible causative microbial agent to be used in the risk assessment of microbial problem buildings is the target in the development of analytical methods, the sampling should be borne in mind from the beginning of the process. The analytical method may work very well in the controlled laboratory circumstances, until the method is tested with environmental samples or in the field conditions. Agents or contaminants in the environment may disturb the analysis and procedures for cleaning of the samples during or after the sampling are needed. The development of the analytical method might have been conducted in a different scale in the lab compared to the concentration range of the agent occurred in the environment, for example the agent occurs at so low concentrations in buildings that it is not possible to analyse from air samples. Thus, close co-operation between researchers developing sampling methods and those developing analytical methods is advisable

Table 1. Comparison of different air sampling methods.

Sampler	Principle	Example of devices	Sampling Capacity	Advantages	Defects
<p>Impactors and Sieve samplers:</p> <ul style="list-style-type: none"> • Spore traps • Slit samplers • Cascade impactors 	<p>Impaction on</p> <ul style="list-style-type: none"> • Agar • Sticky surface • Glass slide • Membranes 	<p>Burkard sampler</p> <ul style="list-style-type: none"> • Air-O-cell cassette • Rotorod sampler • Andersen impactor • SAS • Casella impactor • May impactor • Sierra Marple impactor 	<p>If volumetric:</p> <ul style="list-style-type: none"> • Air flow rate: 2-180 l/min • Sampling time: from minutes to hours, up to a week 	<ul style="list-style-type: none"> • reference data available • some enables long sampling times • some designed for outdoor air sampling • high collection efficiency and low cut-off size (cascade impactors and slit samplers) 	<ul style="list-style-type: none"> • limitations for analytical methods • only for fixed-point sampling • some has short sampling times, not suitable for heavily contaminated environments • drying of collection media • collection efficiency is dependent on air velocity and particle size of microbes (Rotorod, Burkard)
<p>Impingers</p>	<ul style="list-style-type: none"> • Impaction • Centrifugal force • Diffusion into the liquid 	<ul style="list-style-type: none"> • Shipe sampler • AGI-30 • Midget, multi-stage and micro-impingers • BioSampler 	<ul style="list-style-type: none"> • Air flow rate: 0.1-55 L/min • Sampling time: from minutes to hours 	<ul style="list-style-type: none"> • reference data available • suitable for various analytical methods • can be used in a wide range of concentration 	<ul style="list-style-type: none"> • high upper limits • evaporation and foaming of collection liquid during sampling • only for fixed-point sampling • collection efficiency is dependent particle size

Sampler	Principle	Example of devices	Sampling Capacity	Advantages	Defects
Centrifugal samplers	Centrifugal force into <ul style="list-style-type: none"> Liquid Semi-solid Agar 	<ul style="list-style-type: none"> RCS Aerojet cyclone SpinCon 	<ul style="list-style-type: none"> Air flow rate: 40-1000 L/min Sampling time: from minutes to hours 	<ul style="list-style-type: none"> easy to use, low upper limits (RCS) suitable for various analytical methods (SpinCon, Aerojet) enables high volumes, can be used in a wide range of concentration (SpinCon, Aerojet) 	<ul style="list-style-type: none"> high upper limits for cyclones reference data not available (except for RCS) only for fixed-point sampling drying or evaporation of collection media collection efficiency is dependent on particle size
Filter cassette	<ul style="list-style-type: none"> Inertial impaction Interception Sieving onto fibrous, flat or membrane filters (glass fiber, cellulose ester, polycarbonate, Teflon filters) 	<ul style="list-style-type: none"> Burton sampler IOM GSP 	<ul style="list-style-type: none"> Air flow rate: 1-1000 L/min Sampling time: hours 	<ul style="list-style-type: none"> easy to use suitable for various analytical methods enables personal sampling and long sampling times high collection efficiency and low cut-off size 	<ul style="list-style-type: none"> high upper limits filters may contain toxic agents which interfere analyses

AIRBORNE BACTERIA AND FUNGI IN 100 LARGE U.S. OFFICE BUILDINGS

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ABSTRACT

We present preliminary concentrations of airborne bacteria and fungi from the Building Assessment Survey and Evaluation (BASE) study of 100 large U.S. office buildings. Concentrations of total culturable mesophilic (30°C) bacteria were lower indoors than outdoors: range - below the detection limit (DL, 1.3 CFU m⁻³) to 920 CFU m⁻³ and <DL to 6200 CFU m⁻³, respectively. However, concentrations of total culturable thermophilic (55°C) bacteria were similar indoors and outdoors: range - <DL to 230 CFU m⁻³ and <DL to 265 CFU m⁻³, respectively. Median concentrations for these bacteria groups and locations were below DL. Concentrations of culturable fungi also were lower indoors than outdoors: range - 2 to 2045 CFU m⁻³ and 20 to 4150 CFU m⁻³, respectively, medians - 50 and 340 CFU m⁻³, respectively. Concentrations of airborne culturable microorganisms in the BASE study were similar to those observed elsewhere and represent baseline data on U.S. workplace conditions.

INDEX TERMS: airborne bacteria, airborne fungi, office buildings

INTRODUCTION

From 1994 - 1998, the USEPA sponsored BASE-a nationwide, comprehensive study of indoor air and environmental quality, and occupant perceptions of their health symptoms, in 100 randomly selected large office buildings. The overall aim was to acquire representative, baseline information on environmental pollutants and thermal comfort parameters, health symptoms, and building attributes. In this paper, we present selected results from preliminary analyses of the biological measurements.

METHODS

The BASE study systematically measured the indoor and outdoor concentrations of bacteria and fungi in air and source samples and of selected allergens in settled dust. Air samples were collected in the morning and afternoon of the same day at one outdoor and three indoor locations (duplicate samples at the outdoor and one indoor site). Culturable microorganisms were collected with single-stage multiple-hole impactors (Andersen Instruments, Smyrna, GA; $28.3 \pm 1.4 \text{ L min}^{-1}$; 2- and 5-min sampling times). Bacteria were collected on tryptic soy agar (incubated at 30°C and 55°C; 16 outdoor, 32 indoor samples per building). Fungi were collected on malt extract agar (incubated at room temperature; 8 outdoor, 16 indoor samples per building). The laboratory analysts identified seven bacterial groups based on cell shape and Gram-stain reaction and a total of 52 fungal groups (usually identified to the genus level with some *Aspergillus* identified to the species level, Table 1). The minimum detection limits (MDLs) for 2- and 5-min samples, at the level of individually identified and reported groups, were 18 and 7 CFU m⁻³, respectively.

Table 1. Seven groups of culturable bacteria and 52 groups of culturable fungi

Bacteria		
Actinomycetes	Gram-negative cocci	Gram-positive cocci
<i>Bacillus</i> species	Gram-negative rods	Gram-positive rods
		Unidentified
Fungi		
<i>Acremonium</i> spp.	<i>Cladosporium</i> spp.	<i>Pestalotia</i> spp.
<i>Alternaria</i> spp.	<i>Coelomyces</i>	<i>Pithomyces</i> spp.
<i>Arthrinium</i> spp.	<i>Cunninghamella</i> spp.	<i>Rhinoctadiella</i> -like spp.
Arthrospores	<i>Curvularia</i> spp.	<i>Rhizoctonia</i> spp.
<i>Aspergillus flavus</i>	<i>Drechslera</i> spp.	<i>Scopulariopsis</i> spp.
<i>Aspergillus fumigatus</i>	<i>Epicoccum</i> spp.	<i>Sporobolomyces</i> spp.
<i>Aspergillus glaucus</i>	<i>Exobasidium</i> -like spp.	<i>Stachybotrys</i> spp.
<i>Aspergillus niger</i>	<i>Fusarium</i>	<i>Thysanophora</i> spp.
<i>Aspergillus ochraceus</i>	<i>Humicola</i>	<i>Trichoderma</i> spp.
<i>Aspergillus versicolor</i>	<i>Monilia</i>	<i>Tritirachium</i> spp.
<i>Aspergillus versicolor</i>	<i>Nigrospora</i>	<i>Ulocladium</i> spp.
<i>Aspergillus</i> spp. other	<i>Nodulisporium</i> spp.	<i>Verticillium</i> spp.
<i>Aureobasidium</i> spp.	<i>Oedocephalum</i> spp.	<i>Wallemia</i> spp.
<i>Beauveria</i> spp.	<i>Ostrachoderma</i> spp.	<i>Wardomyces</i> spp.
<i>Botryosporium</i> spp.	<i>Paecilomyces</i> spp.	Yeast
<i>Botrytis</i> spp.	<i>Penicillium</i> spp.	Zygomycetes
<i>Calcarisporium</i> spp.	<i>Periconia</i> spp.	<i>Zygosporium</i> spp.
		Unidentified

For further details related to standard operating procedures for environmental measurements and subsequent laboratory and data analyses from sampling conducted in 100 study office buildings - in major cities of 25 states in 10 climate regions - in the winter heating season (48 buildings) and summer cooling season (52 buildings), please refer to USEPA (1994), Womble *et al.* (1999), Macher *et al.* (2004), and Tsai, Macher (2004).

We calculated one indoor and outdoor air concentration for each building by combining morning and afternoon samples as well as 2- and 5-min samples for a volume-weighted average (i.e., total CFU/total air volume). The indoor and outdoor DLs for the composite samples were 0.8 and 2.5 CFU m⁻³, respectively. For the summary statistics presented here, we treated bacterial and fungal values below the DL as zero. We did not use half the DL (0.4 and 1.25 CFU m⁻³) or other value because the number of reported fungal groups varied throughout the study (range 25 to 38 groups). Had we used half the DL rather than zero, the concentration for an indoor sample with 38 groups reported below the DL (47.5 CFU m⁻³) would have been higher than for a sample with 25 groups below the DL (31.2 CFU m⁻³) even though nothing grew from either sample. For consistency, we treated the bacteria data the same in this summary; for more on the bacteria data, see Tsai, Macher (2004).

Airborne fungal spores also were identified from samples collected with a single-stage slit impactor (Burkard Manufacturing Company, Rickmansworth, England; 10 L min⁻¹, 4-min sampling time; two outdoor and six-seven indoor samples per building) in 44 buildings during the last two years of the study (data not presented). Standing water, wet materials, settled dust, dry materials, and surface samples were collected as needed (i.e., where field technicians identified visible evidence of potential microbial growth) for determination of the concentrations of culturable bacteria and fungi (data not presented). The concentrations of cat and dust mite allergens were determined from dust samples collected by vacuuming the floor in the vicinity of one or more of the three indoor sampling locations (further details and data analyses presented in Macher *et al.* (2004)).

For our data management and statistical analyses, the CDHS team consolidated the information from individual data files for biological samples from each building into a single data set. Data management and statistical analyses were conducted using Microsoft Excel and SAS (version 8.2 and Enterprise Guide version 1.3-2, Cary, NC).

RESULTS

Table 2 summarizes the concentrations of culturable bacteria and fungi, using only primary air samples. Table 2A separates bacteria by sampling location and incuba-

tion temperature; Table 2B separates fungi by location. Mean concentrations of both bacteria and fungi were higher outdoors than indoors, by a factor of two for bacteria and seven for fungi. Concentrations were higher both indoors and outdoors for Gram-positive than Gram-negative bacteria. *Cladosporium* species were isolated from 90% of outdoor and 50% of indoor samples. *Penicillium*, *Aspergillus*, and *Alternaria* species and yeasts also were isolated frequently from indoor and outdoor samples. For more information on the occurrence of culturable airborne fungal species inside and outside BASE study buildings, please see preliminary analyses in Womble *et al.* (1999).

Table 2. Concentration of total airborne culturable bacteria and fungi by incubation temperature and location in 100 buildings. NOTE: "90%" was estimated 90% percentile

Category		Concentration (CFU m ⁻³)				
		Mean	Std Dev	Median	Range	90%
A. Bacteria						
Indoors	Mesophiles	15	38	0	0 - 919	50
	Thermophiles	1	6	0	0 - 230	0
Outdoors	Mesophiles	29	149	0	0 - 6184	71
	Thermophiles	2	11	0	0 - 265	0
B. Fungi						
Indoors		99	222	48	2 - 2045	170
Outdoors		706	855	388	20 - 4149	1950

DISCUSSION

The mean, median, and maximum concentrations of airborne culturable fungi (99, 48, and 2289 CFU m⁻³, respectively) were lower in BASE buildings than the corresponding values (300, 82, and >10,000 CFU m⁻³) in a study of 1717 U. S. buildings (Shelton *et al.* 2002). Approximately half (46%) of the buildings in the latter study were office buildings. However, unlike the BASE buildings, the number of samples per building was not uniform (average: 6 versus 16 indoor samples in BASE), and almost half (45%) of the buildings were investigated because of health complaints. The BASE concentrations were similar to but higher than four Boston office build-

ings where airborne fungi were measured every six weeks for a year (mean 42, median 22 CFU m⁻³) (Chao *et al.* 2003). For future reports on fungal concentrations in BASE buildings, we will develop comprehensive lists of culturable fungi from air and source samples to examine the distributions of indoor and outdoor measurements.

In subsequent studies, investigators can use BASE data to predict indoor and outdoor air concentrations and conventional microbial flora, and determine optimal sampling times (ACGIH, 1999; Macher, Burge, 2001). Some bacteria and fungi found in indoor and outdoor air, including some of those assessed in the BASE study (see Table 1), have potential importance because of their allergenicity, association with inflammation, or production of mycotoxins, or as possible indicators of moisture when found indoors (Samson *et al.* 1994; Burge, Ammann, 1999).

CONCLUSION AND IMPLICATIONS

The BASE study of 100 representative large office buildings located in major U.S. metropolitan areas has greatly expanded the available information on typical conditions in office buildings. Preliminary analyses suggested typical U.S. office workers were not exposed to high concentrations of airborne bacteria or fungi. A significant strength of the study design was development of a standardized sampling protocol that was implemented consistently in each of the 100 study office buildings. Indoor and outdoor samples were collected in both heating and cooling seasons, which will allow some investigation of the influences of weather conditions, air filtration, and outdoor air ventilation rates. Following the review of the files, the USEPA will make the final BASE data available to the public and interested researchers.

ACKNOWLEDGEMENTS

This work was funded through a service agreement between the USEPA and the Public Health Foundation Enterprises, Inc. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the agencies supporting this project.

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COMPARISON OF CULTURABLE AIRBORNE FUNGI IN WATER DAMAGED BUILDINGS, BASE BUILDINGS, AND THE OUTDOOR AIR

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ABSTRACT

Air sampling data from six water-damaged buildings from different regions was analyzed for the genera/species and concentration of fungi. Outdoor air samples were collected concurrently with samples from the six buildings. The airborne fungi in water-damaged buildings were compared with the airborne fungi in two control environments, namely the fungi in the outdoor air around the water damaged buildings and the fungi in the indoor air in the U.S. Environmental Protection Agency's BASE (Building Assessment Survey and Evaluation study) buildings from the same geographical area.

Analysis of these data indicates significant difference in the profile of non-phyloplane species and the rank order of culturable fungal species between the indoors and outdoors of water damaged buildings and between the indoor air in the water damaged buildings and indoor air of BASE buildings.

This study shows that the fungal aerosol data available from the BASE study provides a useful baseline against which air sampling results from problem buildings in the same geographical region may be compared.

INDEX TERMS: culturable fungi, BASE, water-damage, mold, air sampling

INTRODUCTION

Environmental samples are commonly interpreted by comparing an environmental measurement with an existing standard. Currently, no standards exist for acceptable concentrations of fungi within indoor air of non-industrial environments even though studies have recognized the association between respiratory symptoms, moisture problems, and fungal growth in buildings (Health Canada 1995; Flannigan, Miller, 2001; Bornehag *et al.* 2001). Commonly the concentration and

composition of fungi within the indoor air are compared to the concentration and composition of fungi within the outdoor air or in control areas in order to interpret air sampling data. The composition of airborne fungi indoors of non-water damaged buildings should, in general, reflect the composition of fungi normally present in outdoor air and should not have a significant difference in rank order of fungi. Thus, phylloplane taxa of fungi such as *Cladosporium*, *Alternaria*, and *Epicoccum* (CAE) (Dillon *et al.* 1999) would be expected to dominate the indoor environment of non-problem dry buildings with no significant difference in the rank order of taxa between indoor and outdoor profiles.

A profile of fungal taxa indoors that differs from that present outdoors or that is dominated by one or two non-phylloplane taxa is generally considered to be an indicator of a moisture or dampness problem. *Penicillium* and *Aspergillus* (PA) have been reported to dominate the indoor environment of buildings with water damaged or moldy building materials (Flannigan, Miller, 2001; ISIAQ, 1996). Comparison of fungal profiles for CAE and PA and rank orders of identified fungi between water-damaged and non-water damaged buildings, and the outdoor air are useful in determining whether fungal amplification is occurring indoors.

The aim of this study is to determine if fungal aerosol data available from the BASE study provides a useful baseline against which air-sampling results from problem buildings in the same geographical region may be compared.

METHODS

BASE buildings: Culturable airborne fungi data from the United States Environmental Protection Agency's (USEPA) Building Assessment Survey and Evaluation (BASE) study were analyzed. The BASE data represents a sample of randomly chosen office buildings (generally thought to be devoid of moisture problems) across the United States. For the purpose of the study the United States was split into 10 climatic regions (signified by the letters A-J) (Womble *et al.* 1996). The BASE culturable airborne fungal data analyzed and presented here were taken from the database of 41 buildings currently available for public use. However, only data from the 26 buildings in BASE regions B, C, D, E, G, and J were analyzed and presented here for comparison to the water damaged buildings.

The method used to collect culturable airborne fungi in the BASE study has been previously published (Womble *et al.* 1996).

Water Damaged Buildings (WDB): Air sampling data from six water-damaged buildings (WDB), one in each of the BASE regions B, C, D, E, G, and J, was analyzed for composition and concentrations of fungi. All buildings had sustained some degree of water damage and subsequent visible mold growth as follows:

1. Region C: This building was a commercial office building which was located partially below grade, and had been flooded several years prior to sampling. Visible mold on walls had previously been removed. Heavy vehicular traffic was consistently present at all times on roads around this building.

2. Region J: This building was a multi-story higher education residence tower where building materials in exterior wall cavities had been damaged by chronic water infiltration, primarily because of leaks around windows. Visible mold growth was hidden in exterior wall cavities. The extent of hidden fungal colonization on gypsum wallboard, insulation, and asphaltic building paper in the exterior walls of each room varied from minimal to extensive according to the ACGIH classification (ACGIH 1999, Chapter 15).

3. Region E: This building was a middle school where building materials in exterior wall cavities had been damaged by chronic water infiltration, primarily around windows and beam penetrations. A moderate amount of visible mold growth was hidden in exterior wall cavities. Building paper and fireproofing material were consistently colonized at water-damaged locations.

4. Region B: This building was a multi-unit residence building where a moderate amount of visible mold was present in several rooms of the below grade level areas of the building. Visible mold was present on gypsum board walls, kraft paper of fiberglass insulation and carpet surfaces. Visible mold was not observed on interior surfaces in above grade levels of the building.

5. Region G: This building was a research office building where a flood thoroughly soaked gypsum board firewalls in one wing of this building while a second wing was unaffected by the flood water. Mold grew on paper facers of water soaked gypsum board firewalls in the water-damaged wing. The outermost, but not the innermost gypsum board layers were removed during restoration. Consequently, hidden mold was left in the wall cavities of the water-damaged wing.

6. Region D: This building was a multi-unit residence building where, as a consequence of firefighting, water thoroughly soaked building materials. Mold grew on most biodegradable building materials. During clean up, mold was physically removed from accessible portions of the building. Sampling occurred at a pre-occupancy stage of the restoration (prior to vacuuming with high efficiency particulate air (HEPA) filtered vacuum equipment).

Air sampling within the water-damaged buildings was performed using a portable culture plate impactor (PBI, Surface Air System, 219 jets) operating at a flow rate of approximately $0.180 \text{ m}^3\text{min}^{-1}$. Samples were collected on malt extract agar (MEA) and cellulose agar media prepared according to the American Industrial

Hygiene Association's (AIHA) Field Guide (AIHA 1996). Outdoor air samples were collected on the roof of the building, or where the roof was not accessible, at grade level at least 8 meters from the building envelope. Sampling was not carried out at times of the year when snow was on the ground (AIHA 1996).

Indoor and outdoor samples from BASE buildings and the WDB mentioned above were analyzed in a laboratory by determining the total colonies of each genus and, in the case of *Aspergillus*, each species by morphological identification. Mean concentrations of each genus and/or species, total airborne fungi, CAE, and PA were then calculated (concentrations below the detection limit were assigned a zero) for each building (BASE and water-damaged) and outdoor air.

Rank order frequency of each identified genus and species (*Aspergillus* only) was calculated using the pooled data from each of the buildings and were compared using a non-parametric rank order correlation test (Spearman's Correlation Test) to determine if a correlation exists between the rank order of indoor air in WDB, in indoor air in BASE buildings, and in the outdoor air (ACGIH 1999, Chapter 13).

RESULTS

Fungi in outdoor air around WDB: The average concentrations of total culturable fungi, CAE, and PA in outdoor air around WDB are shown in Table 1. In the outdoor air around the WDB, CAE were more prevalent than PA at all locations. The PA levels in the outdoor air were somewhat higher in the outdoor air in the southwest regions (Regions J & E) as compared to midwest (Regions B & C) and southeast (Regions D & G) locations. *Cladosporium* was always the most abundant genus present in the outdoor air at all locations. CAE comprised 54 to 91 % of the total average culturable fungal concentration. The highest percentage of PA concentration was in southwest Region J (12%).

Fungi in indoor air in WDB: The average concentrations of total culturable fungi, CAE, and PA in indoor air of the WDB are shown in Table 1. In the indoor air of the WDB, the PA level was higher than that of CAE in three of the buildings [Regions C, B, & G (Flooded Wing)]. In the building in the region G the PA level was markedly higher in the flooded wing as compared to the unflooded wing (1100 CFU/m³ versus 170 CFU/m³, respectively; Table 1). In all regions the PA concentration both on a percent and an absolute basis was higher in indoor air versus outdoor air.

The building in Region B was the only water damaged building where culturable *Stachybotrys* was consistently present in the indoor air (average concentration of 15 CFU/m³).

Although detailed analysis is not presented here, *Aspergillus versicolor* was the most common *Aspergillus* species found in the indoor air of WDB. *Aspergillus niger* was the most common species in the outdoor air.

Fungi in outdoor air around BASE buildings: The average concentrations of total culturable fungi, CAE, and PA in outdoor air around BASE buildings are shown in Table 2. In the outdoor air around the BASE buildings CAE were more prevalent than PA at all locations. The PA levels were somewhat higher in the outdoor air in the southwest regions (Regions J & E) as compared to midwest (Regions B & C) and southeast (Regions D & G) locations. Thus, fungi in the outdoor air around BASE buildings were qualitatively similar to those found around WDB.

Fungi in indoor air in BASE buildings: The average concentrations of total culturable fungi, CAE, and PA in indoor air in BASE buildings are shown in Table 2. The total average concentration of fungi was higher in outdoor air than in indoor air in all regions. The average PA level was lower than the average CAE level in the indoor air in five of the six regions (exception is Region J). In Region J, PA and CAE comprised 41 and 36% of the total concentration, respectively. The lowest concentration of CAE indoors in BASE buildings on a percentage basis was in Region D (24%).

Correlation: The pooled taxa from all air samples collected from outdoor and indoor air in WDB, and BASE buildings were rank-order compared using Spearman's Correlation Test (Table 3). Significant correlation was found between the rank orders of identified fungi in outdoor air around BASE buildings and in indoor air of BASE buildings in all regions. Significant correlation between the rank orders of identified fungi in outdoor air around water damaged buildings and in indoor air of WDB was found only in Regions J and E. Significant correlation was not found between the rank orders of identified fungi in indoor air in WDB and in indoor air of BASE buildings in any of the regions.

Table 1. Culturable fungi concentrations in indoor and outdoor air at WDB.

Geographical Region	Sample Location	# of samples	<u>Average Concentration (CFU/m³)</u>		
			Total Mean	CAE (%)	PA (%)
Region C	Indoor Air	19	570	25	71
				(4)	(12)
	Outdoor Air	10	4400	4000	3
				(91)	(<1)
Region J	Indoor Air	97	1400	650	450
				(46)	(32)
	Outdoor Air	18	510	390	60
				(76)	(12)
Region E	Indoor Air	159	630	510	100
				(81)	(16)
	Outdoor Air	43	770	680	41
				(88)	(5)
Region B	Indoor Air	20	430	31	180
				(7)	(42)
	Outdoor Air	20	550	300	24
				(54)	(4)
Region G	Indoor Air (Flooded Wing)	38	1900	220	1100
				(12)	(58)
	Indoor Air (Unflooded Wing)	38	1300	460	170
				(35)	(13)
	Outdoor Air	20	540	460	29
				(85)	(5)
Region D	Indoor Air	24	320	170	74
				(53)	(23)
	Outdoor Air	12	310	260	13
				(84)	(4)

Table 2. Culturable fungi concentrations in indoor and outdoor air at BASE buildings.

Geographical Region	Sample Location	# of samples	Average Concentration (CFU/m ³)		
			Total Mean	CAE (%)	PA (%)
Region C	Indoor Air	80	44	20	7
				(45)	(16)
	Outdoor Air	40	320	190	70
				(59)	(22)
Region J	Indoor Air	192	59	21	24
				(36)	(41)
	Outdoor Air	96	590	350	150
				(59)	(25)
Region E	Indoor Air	49	170	99	13
				(58)	(8)
	Outdoor Air	23	1100	560	83
				(51)	(8)
Region B	Indoor Air	272	68	31	10
				(46)	(15)
	Outdoor Air	134	650	450	15
				(69)	(2)
Region G	Indoor Air	79	38	14	11
				(37)	(29)
	Outdoor Air	40	440	260	24
				(59)	(5)
Region D	Indoor Air	271	90	22	15
				(24)	(17)
	Outdoor Air	133	760	340	56
				(45)	(7)

Table 3. Results of Spearman’s correlation test of rank orders of identified fungal colonies between indoor air in WDB, indoor air in BASE buildings, and outdoor air.

Comparison	Geographic Regions					
	C	J	E	B	G	D
BASE Indoor Air vs. BASE Outdoor Air						
WDB Indoor Air vs. WDB Outdoor Air	X			X	X ¹	X ²
BASE Indoor Air vs. WDB Indoor Air	X	X	X	X	X	X

“X” identifies NO significant correlation (at $p = 0.05$) between rank order of species using Spearman’s Correlation Test; ¹Flooded Wing; ²Unflooded Wing

DISCUSSION

In this study the airborne fungi in water-damaged buildings were compared with the airborne fungi in two control environments, namely the fungi in the outdoor air around the WDB and the fungi in the indoor air of BASE buildings from the same geographical area.

Comparison of the indoor air of WDB and the indoor air of BASE buildings showed that the PA level in all WDB was consistently higher by at least 100% than the PA level in BASE buildings in all geographical regions. The average PA level (330 CFU/m³) in WDB was approximately 25 times higher than the average BASE PA level (13 CFU/m³). Comparison of rank order of identified fungi indicated a significant difference between WDB and BASE buildings in all regions. Rank order comparisons however did not consider the time of year or time of day that samples were collected and this may affect the effectiveness of these comparisons.

BASE data for each region was collected from several buildings within each geographical zone. The comparable data for WDB was collected from only a single building within the region. Comparisons of the WDB and BASE buildings might be more meaningful if more WDB in each region were included. Also the analysis of the data was limited by the amount of BASE building data currently available. Data from only 26 of the 41 BASE buildings was available for comparison.

Comparison of rank order of identified fungi was limited by the absence of specification of *Penicillium* and other genera in the BASE data.

Three of the WDB contained hidden mold in exterior wall envelopes. In two of these buildings (Region J and Region G) the PA levels indoors were at least five times higher than those outdoors suggesting that some PA spores from the hidden mold colonization was entering the indoor air. However, in the third WDB in Region E, the PA level indoors was less than that outdoors. In this building, at the time of sampling, PA spores associated with the hidden growth (e.g., *Penicillium chrysogenum*) within wall materials did not enter the indoor air. This occurrence in a building with hidden mold growth (Morey *et al.* 2002) suggested that factors that were not investigated in the study, such as air pressurization differences (Airaksinen *et al.* 2004) between the wall envelope and interior spaces could affect the entry of spores (on air currents) and consequently the sampling results from WDB.

CONCLUSION AND IMPLICATIONS

Fungal aerosol data available in the BASE study provides a useful baseline against which air sampling results from problem buildings in the same geographical area may be compared. For example, PA levels were consistently higher in WDB as compared to BASE buildings. Comparison of rank order correlation between BASE and WDB indicated significant difference in five of six regions and may be a useful tool in comparing the buildings. More useful correlations may be obtained when data from BASE buildings is matched with data from WDB under similar environmental conditions (i.e., winter/summer).

It is widely recognized that in indoor mycological investigations sampling is secondary to a thorough visual inspection of building construction. A competent visual inspection of the building remains a critical component when determining whether fungal colonization exists within a building (Health Canada 1995; ISIAQ 1996; NYC, 2000; EPA, 2001; AIHA, 2001). The WDB from Region E had a relatively low indoor PA level (lower than outdoors), the rank orders of fungal genera indoors and outdoors were not significantly different, and the rank orders of *Aspergillus* species were similar, even though destructive inspection in this building showed extensive evidence of water incursion that resulted in moderate amounts of mold colonization including *Penicillium* and *Aspergillus* species within the exterior wall cavities. Pressurization relationships of the type described by Airaksinen *et al.* 2004 may have been responsible for keeping *Penicillium* and *Aspergillus spp.* spores hidden in wall cavities from entering indoor air.

Alternative indoor sources of PA (such as contamination of food or fungal growth in potting soil) not related to water damaged building materials can also have an impact on air samples. Thus, air sampling data from any building should always be cautiously interpreted in conjunction with results from an informed inspection of building construction.

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SHARED AIR: EXAMINING THE CONTRIBUTION OF MOLD FROM HOME CRAWL SPACES TO HOME INTERIORS

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ABSTRACT

One potential source of a “damp” home is crawl space construction. In recent studies we assessed mold contamination levels within crawl spaces and characterized whether air leakage from the heating, ventilation and air conditioning (HVAC) system, and associated ductwork, transports viable mold spores from the crawl space into the living spaces within the home. The sampling protocol involves the use of an Andersen two-stage impaction sampler to collect viable mold spores from the crawl space, the interior of the home, “supply” air coming directly from the HVAC system, and the outdoors. Results from 77 homes confirmed that crawl spaces are potentially important reservoirs of mold species that appeared to be transported into the occupiable areas of the home environment in 31% of the houses investigated. Transmission in these homes was characterized by significantly higher levels of mold spores in the crawl space, in the indoor air when the HVAC system was running, and in air samples collected at the supply air diffusers. In addition, the rank order of the fungal species with the HVAC system running shifted to reflect the dominant species present in the crawl space sample. The HVAC system was identified as a vehicle for transmission of this contamination from the crawl space into the living spaces. Potential causes of the mold growth and spore distribution appear to be inadequate insulation of cold surfaces in the crawl space, leading to extensive condensation, and air leakage in the HVAC system and ductwork.

INTRODUCTION

One potentially important source of mold and moisture in the home environment is the crawl space. Crawl spaces represent a dominant construction type in the Southeastern United States. For more than a century, homebuilders have built

crawl spaces with wall vents on the premise that these vents keep crawl spaces dry by dissipating excess moisture accumulation to the outside air. While passive ventilation of crawl spaces through the use of wall vents may work in theory, researchers have noted that many wall vented crawl spaces have serious moisture problems. A chief finding reported at the American Society of Heating, Refrigeration, and Air Conditioning Engineers (ASHRAE) Symposium (Recommended Practices for Moisture Control in Crawl Spaces) was that wall vented crawl spaces in existing homes are often dangerously wet [Rose W. B. 1994]. Similar moisture problems associated with crawl space construction have been identified in Scandinavia and other parts of the world [Wickman, W. *et al.* 1994; Emenius, G. *et al.* 2004].

Heating and cooling systems and associated ductwork are typically located in crawl spaces. According to the U.S. Environmental Protection Agency, residential duct systems typically leak 15 to 20 percent of the air they convey. Of particular concern is return duct air leakage. In crawl spaces, return duct leakage can draw contaminants such as dirt, dust, mold, radon and pesticides into homes [U.S. Environmental Protection Agency, 2000; Airaksinen, M *et al.* 2004]. In addition, leakage from of air-conditioned air from supply ducts can cause extensive condensation from the moist, warm outdoor air that infiltrates the crawl space.

In a wall vented, crawl space home, the sub and finish floor materials combine to form an air barrier that separates the crawl space and home environments. Breeches in the floor boundary between the crawl space and the home are common. Typical construction practice makes unsealed, penetration holes for the electrical, plumbing, ductwork, phone and cable lines that cross the floor barrier. Further breeches result from seam and edge gaps in the sub and finishing flooring materials. Physics dictates that air leakage events will occur through duct and floor holes whenever any combination of wind, stack effect, and house fan use results in air pressure differences between the home and the crawl space. The resulting air communication may lead to the eventual transport of mold species to the livable part of the home environment.

The aim of our study was to assess the mold contamination levels within crawl spaces and characterize whether air leakage from the HVAC system, and associated ductwork, transport viable fungal spores from the crawl space into the living spaces of the home.

METHODS

We established a sampling protocol designed to determine whether crawl spaces serve as reservoirs for transportation of mold species by the HVAC system to the livable part of the home environment. The sampling protocol included indoor and

outdoor temperature and relative humidity readings. Interior and exterior bioaerosol samples for respirable fungal spores were collected in 77 homes to assess the concentration of aerosolized fungal spores.

The homeowner agreed not to run the crawl space heating, ventilation, and air-conditioning (HVAC) system for four hours prior to the sampling. Two sets of samples were taken during the test. First, before the HVAC system fan was turned on, three samples were taken: one near the “return” grill for the HVAC system, one in the crawl space, and one outside the house. Then, the system fan was turned on and allowed to run for at least 5 minutes before two additional samples were taken, one near the return grill and one at a supply air diffuser (or register). The supply diffuser sample was collected inside a short polyethylene tube that is temporarily taped around the supply register. The tube isolates the supply air from potential contaminant sources within the house, thus allowing characterization of the relative contribution of the HVAC system to the total bioburden within the house.

This sampling was conducted by two trained indoor air quality technicians using Andersen two-stage cascade impactors, which collect and separate both non-respirable and respirable size particles. The sampler was connected to a vacuum pump calibrated to collect air samples at the rate of 28.3 L/min (one cubic foot per minute). Equipment calibration was conducted at the beginning of sampling, at mid-day, and at the end of the day. A sampling period of 3.5 minutes was used for the outdoor air sample and all samples collected within the houses. The sampling period for the crawl space samples was 1.0 minute. The collection medium used for impaction of fungal spores was Malt Extract Agar, an aciduric mycologic medium designed for the collection of environmental fungi. After sampling, the culture plates were transported back to Duke University and incubated at ambient temperature for 96 hours prior to enumeration and identification. The samples were protected from temperature extremes during transportation. Fungal identification was accomplished by macroscopic examination of colony morphology and microscopic examination of fungal elements. Bioaerosol samples provide an evaluation of the total number of breathable fungal spores, reported in colony forming units per cubic meter of air, and the most common species of mold found.

All statistical analyses were conducted using the StataSE 8 software program. We employed two-sided ttests to determine whether the levels of fungi at each point in the sampling protocol were different across homes that were characterized as “transmission” versus “non-transmission”.

RESULTS AND DISCUSSION

In order to determine if transmission was occurring from the crawl space to the livable part of the home environment, we examined two critical conditions. First, we assessed the impact of the HVAC system on the indoor environment by comparing the concentrations of viable fungal spores from indoor air with the HVAC system off with the concentration when the HVAC system was turned on, as well as with the concentration in the diffuser airstream. If there was a significant increase in the fungal spore concentration in the latter two samples, the first indicator of potential transmission (Condition 1) was considered to be satisfied. The second criterion (Condition 2) for determining potential transmission was the mix and rank order of the dominant mold species in all of the bioaerosol samples. In order for Condition 2 to be satisfied, there had to be a shift in the mix and rank order of the indoor samples with the HVAC system running to reflect the dominant mold species present in the crawlspace sample, with that rank order being different from the outdoor sample. The statistical analysis of the changes in fungal species among the various sample locations has not been finalized and is not included in this paper. However, *Penicillium* species and/or *Aspergillus* species were the predominating mold species in the crawl space samples when the crawl space rank order was significantly different than the outdoor conditions. These same species of fungi predominated in both the indoor samples with the HVAC system on and the diffuser samples in the homes where Condition 2 was satisfied.

If both Conditions 1 and 2 held, then transmission was determined to be occurring (T). If neither condition held, then no transmission was determined to be occurring (NT). If only one condition held, then we were not able to determine definitively whether transmission was occurring (NT/T~). Table 1 below provides some summary statistics on the three different categories to which we assigned homes.

While levels alone do not tell the full story, it is interesting to note the significantly higher levels of fungi in the crawl space ($p=.001$), indoor with HVAC on ($p=.003$), and diffuser samples ($p=.06$) were measured in “transmission” homes with Condition 1 and 2 as compared to the non-transmission homes. Note also that the indoor samples with the HVAC system off have indistinguishable means between the transmission and non-transmission homes.

Table 1. Summary statistics on sample homes

			Fungal spore counts (CFU/m ³)			
	Sample	# Observ.	Mean	Std.Dev.	Min.	Max.
No transmission homes (NT)	Outdoor	38	2609	3814	124	11756
	Crawl space	38	12846	16140	105	41146
	Indoor (HVAC off)	38	631	520	51	2510
	Indoor (HVAC on)	38	546	406	82	1962
	Difuser	38	373	494	10	3074
Transmission homes (T)	Outdoor	24	3895	4343	585	11756
	Crawl space	24	33678	13558	3633	41146
	Indoor (HVAC off)	24	676	599	146	2408
	Indoor (HVAC on)	23	1132	698	244	3122
	Difuser	23	1101	1413	267	5993
Indeterminate (NT/T~)	Outdoor	15	3974	4719	51	11756
	Crawl space	15	29720	16959	935	41146
	Indoor (HVAC off)	15	1836	3843	30	11756
	Indoor (HVAC on)	15	2357	3925	102	11756
	Difuser	15	1108	2375	82	11756
All homes	Outdoor	77	3285	4213	51	11756
	Crawl space	77	22164	18312	105	41146
	Indoor (HVAC off)	77	1015	2231	30	11756
	Indoor (HVAC on)	76	1210	2306	82	11756
	Difuser	76	739	1511	10	11756

This preliminary analysis indicates that the crawl space is a significant potential reservoir for the amplification of mold. The HVAC system clearly served as a vehicle for transmission of this contamination from the crawl space into the living spaces of 31 percent of the houses sampled. In only 49 percent of the homes were we able to rule out transmission entirely. These results call out for additional data collection and more thorough analysis of characteristics of crawl spaces and HVAC systems that make transmission more likely.

CONCLUSIONS AND IMPLICATIONS

This preliminary analysis indicates that the crawl space is a significant potential reservoir for the amplification of mold. The HVAC system clearly served as a vehicle for transmission of this contamination from the crawl space into the living spaces of 31 percent of the houses sampled. In only 49 percent of the homes were we able to rule out transmission entirely. Based on our observations, potential causes of the mold growth and spore distribution appear to be inadequate insulation of cold surfaces in the crawl space, leading to extensive condensation. In addition, leakage of air-conditioned air from supply ducts appeared to have caused extensive condensation from the moist, warm outdoor air that infiltrates the crawl space. These results call out for additional data collection and more thorough analysis of characteristics of crawl spaces and HVAC systems that make transmission more likely.

ACKNOWLEDGMENTS

The authors wish to acknowledge Gamaliel Whitney, and Carolina Pungiluppi-Tono for their excellent work in collecting the field data; as well as Michelle Abrams and Kelly Atkinson for data management.

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MODELING THE EQUILIBRIUM SPORE LOAD FOR A BUILDING

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ABSTRACT

Many factors influence the levels of mold spores in buildings. We describe a method for modeling the equilibrium spore load for a building assuming the air exchanges per hour (ACH) and average outdoor concentrations to be constant. The model includes considerations for infiltration, exfiltration, settling, entrainment, tracking, cleaning and building filtration. Although these conditions are almost never static, the model permits a better understanding of how spore deposition, on surfaces and the spore load in the air in buildings, react to changing conditions. The model allows the user to select and test different unknown conditions in order to follow the patterns that develop between the variable relationships.

INDEX TERMS: Model, Equilibrium, Mold, Spore Load, Spore Deposition

INTRODUCTION

The indoor air and surface concentrations for mold spores in a building are determined by a complex set of interactions between the outdoor concentrations, occupant activity and building dynamics. Mold spores can enter a building through a number of ways including air infiltration, tracking, and colonization of materials. Mold spores can exit a building through exfiltration, filtering, and various cleaning activities. Mold particulates may also deposit onto surfaces by settling from the air, and become airborne by entrainment due to various activities (Macher 1999). In order to develop a model to describe the equilibrium spore load for a building and understand its functions and limitations, each of the factors affecting mold particulate levels in buildings needs to be considered.

METHODS

In general the formula for total spores in a building is:

$$(1) \quad [Spores_{total}] = [Spores_{start}] + [Spores_{entering}] - [Spores_{exiting}]$$

The model is based on the premise that all spores present in a building will either be in the air or on surfaces. As spores settle from the air, they are subtracted from the air concentration and added to the surface concentration. As spores become airborne by entrainment, they are subtracted from the surface concentration and added to the air concentration. Throughout this process, the effects of air infiltration and exfiltration continue. The total spores in a building can be broken further into a formula representing spores present in the air, and another formula representing spores present on surfaces.

The general formula for airborne spore concentration is:

$$(2) \quad [Spores_{air\ start}] + [Spores_{infiltration}] - [Spores_{settling}] \\ + [Spores_{entrainment}] - [Spores_{exfiltration}]$$

The general formula for the surface spore concentration is:

$$(3) \quad [Spores_{settled\ start}] + [Spores_{settling}] - [Spores_{entrainment}] \\ + [Spores_{tracking}] - [Spores_{cleaning}]$$

Spores_{air start} is an assigned number for the concentration of airborne spores present indoors at the beginning of the study period. This number is based on measured indoor conditions or conditions to be tested.

Spores_{infiltration} is the calculated concentration of spores that enter the building during the study period and is based on air exchange, building filtration and the outdoor spore concentration. The outdoor concentration is an assigned number based on measured outdoor concentrations or conditions to be tested.

Spores_{settling} is the calculated concentration of spores that settle from the air to surfaces in the building during the study period.

Spores_{entrainment} is an assigned concentration of spores based on the percentage of spores that are released from surfaces into the air during the study period.

Spores_{exfiltration} is the calculated concentration for the airborne spores that leave the building during the study period and is based on the air exchange and the indoor concentration.

Spores settledstart is the assigned concentration for the spores on surfaces in the building and is based on measured indoor conditions or conditions to be tested.

Spores tracking is the assigned concentration of spores brought into the building by the occupants or their activities.

Spores cleaning is an assigned concentration based on the percentage of spores removed by the occupants or their direct or indirect activities.

The concentration of spores present at any given minute will affect the concentration of spores that will be present in the next minute. This requires the use of a formula that involves iterations. A starting point must be chosen. The quantity of spores settling is based on concentrations present in the air at the beginning of the iteration. If a sufficiently short iteration period is chosen, the error introduced by not including the change in spore concentrations during the iteration period should not be significant. For this model an iteration period of 1.0 minute has been chosen. During iterations, the change in spore concentrations from conditions such as infiltration, exfiltration, settling and entrainment from the previous minute are considered by adding or subtracting them from the appropriate variable for the next iteration minute. An appropriate conversion factor must be used to convert between the airborne and surface concentrations.

The model does not consider individual taxa of mold spores. The model is based on spore size which is assigned by the user. By assigning a specific spore size for a fungal organism this model can be used to track changes for specific organisms.

Every building will have some level of air exchange with the outside environment. The number of ACH is determined by the amount of deliberate ventilation with the outside through design factors such as mechanical equipment, as well as, incidental infiltration from “leaks” around windows, doors and other parts of the building envelope. Occupants may also contribute to ventilation by leaving open doors and windows. Mechanical ventilation provides a controlled infiltration through intended openings, but other forces such as stack effect and wind blowing on the outside of a building can result in significant air exchange through other available openings (Lstiburek 1994). Winds as low as one mile per hour have been demonstrated to result in significant air exchange in buildings (Burge 1995).

The concentration of spores entering the building from outside air may be reduced by intentional filtration in HVAC systems (Kemp, 2003). Filtration by the building materials, as air enters by infiltration through building components, may also reduce the concentrations of spores entering a structure. This can be represented by the following formula:

$$(4) \quad [Spores_{entering}] = (Coef_{filtration}) [Spores_{outside}] \frac{(ACH)}{60}$$

The coefficient of filtration is an assigned number between zero and one. A coefficient of zero represents perfect filtration (i.e., concentration of spores entering the building is zero), whereas a coefficient of one represents no filtration reduction of spores that will enter the building (i.e., concentration of spores entering the building is equal to the concentration of spores outside).

The differing sizes and shapes of various species of mold spores determine their settling rates. The settling rate for different mold spores can be determined by applying Stokes' formula for particle settling rates. Stokes' formula demonstrates that mold spores with the same density will settle at a rate proportional to the square of the mean aerodynamic radius (r) of the particle (Flannigan, 2001).

$$(5) \quad V = \frac{\sigma - \rho}{\mu} g(r)^2$$

Where V is the terminal velocity in cm/sec, σ is the density of the spore, ρ is the density of air, μ is the viscosity of air (1.8×10^{-4} g/cm sec), g is the acceleration due to gravity (981 cm/sec) and r is the radius of the spore measured in cm. Fungal particle densities common in the outdoor environment have been reported to have a density ranging from 0.56 to 1.44 grams/cm (Reponen 1995). For purposes of this model the density of fungal spores is assumed to be 1.0 and the density of the air is not considered significant for these calculations (Flannigan, 2001). For this model Stokes' formula with velocity in meters/min has been simplified to:

$$(6) \quad V = 1.2 \times 10^4 (r)^2 \times 60$$

This demonstrates that for the assumptions made in this model, a 10 μ mold spore will settle one hundred times faster than a 1.0 μ mold spore, and approximately ten times faster than a 3.0 μ mold spore.

As occupants move around in buildings, they will stir up particles causing an entrainment of spores into the air from surfaces. The amount of activity, type of surface and other factors determine the entrainment that will occur (Asbury, 2002).

$$(7) \quad [Spores_{entrainment}] = (Coef_{activity}) [Spores_{settled}]$$

The amount of mold spores that will exit a building is dependent on the air exchanges per hour and the concentration of airborne spores available to travel to the outside.

$$(8) \quad [Spores_{exfiltration}] = [Spores_{air\ start}] \frac{(ACH)}{60}$$

Additional spores will be tracked into the building on the bottom of shoes, on clothing, by pets and other sources (Cole 1999). The amount of spores brought into the indoor environment is dependent on the number of people living in the structure, the frequency of entry, the fungal load on the surfaces encountered and lifestyle habits, such as removing ones shoes prior to entry. For the purpose of this model, the spores tracked into the building will be a variable concentration that can be changed to offer insight into how a building might react to various scenarios. Although it is recognized that spores brought in by animals and on clothing may become airborne, most tracked mold is likely to be transferred directly to building surfaces. For the purpose of this model, it will be assumed that all of the tracked spores will be deposited on surfaces and not immediately entrained into the air.

$$(9) \quad (Coef_{tracking}) [Spores_{settled}]$$

Various activities, such as cleaning by vacuuming, have been demonstrated to have varying effectiveness (Anderson 1969). For the purposes of this model, it will be assumed that the only spores that can be removed by cleaning are those that have landed on surfaces and not those that are airborne.

$$(10) \quad (Coef_{cleaning}) [Spores_{settled}]$$

Spores may also exit the building due to other miscellaneous factors, such as efficient re-circulated HVAC filtration or portable air filtration devices. Additional spore removal could be calculated using the filtration efficiency and the ACH for the equipment.

At the end of the first minute, the indoor air concentration of spores of a chosen size is calculated by the following formula:

$$(11) \quad [Spores_{air\ start}] + (Coef_{filtration}) [Spores_{outside}] \frac{(ACH)}{60} \\ - [Spores_{air\ start}] \left(\frac{1.2 \times 10^4 (r)^2}{height} \right) \cdot 60 + \\ (Coef_{activity}) \left(\frac{Spores_{settled\ start}}{height} \right) \\ - [Spores_{air\ start}] \left(\frac{ACH}{60} \right) = [Spores_{air\ end}]$$

Where the term “height”, is the height of the room in meters.

At the end of the first minute, the indoor spore load for surfaces with spores of a chosen size is calculated by the following formula:

$$(12) \quad \begin{aligned} & [Spores_{settledstart}] + [Spores_{airstart}] \left(1.2 \times 10^4 (r)^2 \right) 60 \\ & - (Coef_{activity}) [Spores_{settledstart}] + (Coef_{tracking}) [Spores_{settledstart}] \\ & - (Coef_{cleaning}) [Spores_{settledstart}] = [Spores_{settledend}] \end{aligned}$$

For the first minute, $Spores_{settledstart}$ and $Spores_{airstart}$ are the assigned starting value for equations (11) and (12). $Spores_{settledend}$ and $Spores_{airend}$ are the ending values at the end of the first minute. For the next minute and each succeeding iteration the $Spores_{airend}$ value is substituted for the $Spores_{airstart}$ and the $Spores_{settledend}$ is substituted for the $Spores_{settledstart}$. The iterations are continued for the total number of minutes to be studied.

As spores enter or leave the air, its concentration units change. Equations (11) and (12) require that concentrations for settling and entraining spores are converted by using an appropriate factor when adding them into the equations.

DISCUSSION

In order to utilize this model, it is necessary to make several assumptions.

The air within the building is perfectly mixed, so there is an equal concentration of mold spores in the air throughout the entire building.

An iteration time of one minute provides a small enough time unit of measure to observe equilibrium trends between the mold spore concentrations in the outside air, the inside air and on surfaces.

The concentration of mold in the outside air is constant and uniform throughout the modeling.

When considering the fungal component of bioaerosols that may be present, it is important to realize that the mold particles may be viable or non-viable.

This model does not differentiate between viable and non-viable spores, or different kinds of mold. It is important for the user of the model to be consistent with the data that is input. In order to consider organism types, the user must assign a spore size to the organism. It is recommended the size assigned be consistent with published data recognizing that there is inherent size variability even within the same species (Reponen 1995).

Some interesting equilibrium dynamics develop between airborne mold particles and surfaces with settled spores. This model supports previously reported research demonstrating equilibrium levels of spores on surfaces develops in normal buildings over the course of approximately thirty days (Anderson 1969).

Spores that settle leave the air, but do not leave the building unless they are removed by cleaning. Settled spores can also be entrained into the building's air and removed by exfiltration at a later time.

CONCLUSIONS

This model has been developed as a teaching tool to help students understand the multiple and variable dynamics that affect mold levels within indoor environments. It allows students to explore various airborne and surface mold scenarios. By manipulating one variable at a time and comparing results it is possible to observe outcomes predicted by the model. The application of this model demonstrates that the difference between indoor and outdoor air mold counts in normal buildings is primarily due to settling or collection of the spores on interior surfaces rather than by exfiltration to outdoor air. For example the model predicts that, in still air, most *Cladosporium* and other larger spores will settle to the floor before normal air exchange can move them out of the building.

Over time, given constant outdoor concentrations, equilibrium develops between indoor and outdoor air and a second equilibrium develops between the concentrations of spores that settle and those that are entrained.

Further research is required to validate this model and the interpretation of the predicted results. Further understanding of building fungal equilibrium dynamics should assist with model improvements. Additional field and statistical analysis work is required to determine if model predictions are comparable to measured data.

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RELEASE OF *ASPERGILLUS VERSICOLOR* FRAGMENTS AND SPORES FROM CONTAMINATED SURFACES

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ABSTRACT

Fungal cultures of *Aspergillus versicolor* of three different ages (28~32-weeks old (old); 8~9-weeks old (mid-aged); 4~5-weeks old (young)) were used as the fungal sources. Fungal propagules were released into the air by Fungal Spore Source Strength Tester (FSSST), fractionated according to their aerodynamic sizes and measured using an Electrical Low-Pressure Impactor (ELPI). A bimodal size distribution of airborne fungal propagules was observed for all three different aged fungal cultures. One mode was in the size range of 1.26-1.99 μm representing intact spores, and the other was in the range of 0.07-0.31 μm representing fragments. The fragment-spore ratio and the calculated spores size was affected by fungal age showing the smallest spore size and highest fragment-spore ratio for the old culture and the largest spore size and lowest fragment-spore ratio for the mid-aged culture. These results will be used in developing a field-compatible method for the sampling of fungal fragments.

INDEX TERMS: Fungal fragments, Size distribution, Impactor

INTRODUCTION

Epidemiological studies have shown that people living or working in mold problem buildings have more respiratory symptoms and diseases than people in non-problem buildings (Husman 1996; Dearborn *et al.* 1999; Etzel *et al.* 1998; Meklin *et al.* 2002). However, a clear cause-and-effect relationship has not been well established between the health effects and the concentrations of airborne fungal spores. Several field studies have shown that the concentration of airborne fungal spores in mold problem buildings is not necessarily higher than in non-problem buildings (Cooley *et al.* 1998; Hyvärinen *et al.* 1993; Nevalainen *et al.* 1991; Strachan *et al.* 1990).

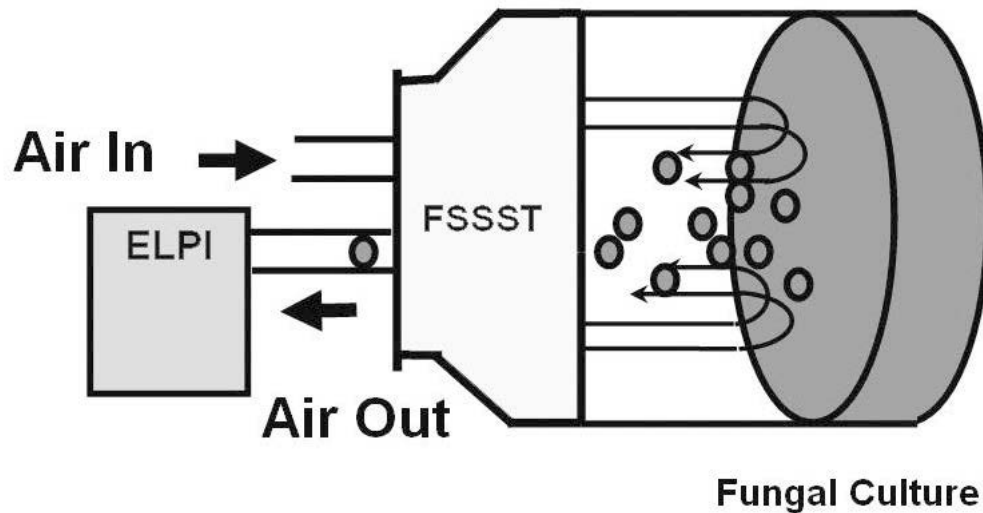
Therefore, other types of fungal-related exposures need to be explored more. In our earlier investigations on the release of fungal spores, performed in the controlled laboratory environment, we found that smaller-sized fungal fragments are released together with spores from contaminated surfaces in large quantities (Górny *et al.* 2002). Fungal fragments have become particularly interesting in the light of recent epidemiological studies that have reported strong relationship between fine particles and adverse health outcomes (Dockery *et al.* 1993; Magari *et al.* 2001; Magari *et al.* 2002; Pope *et al.* 1999; Pope 2000; Gold *et al.* 2000; Pekkanen *et al.* 2002). We hypothesize that fungal fragments may be potential source contributing to the adverse health effects. The purpose of this study was to confirm the release of fine fungal fragments from mold-contaminated surfaces and to characterize the size distribution using an advanced technique based on simultaneous real-time measurement and collection of fine particles.

METHODS

Fungal species and test material: Pure fungal colonies of *Aspergillus versicolor* on Malt Extract Agar (MEA) plates were scraped from the agar plate, mixed with deionized and sterilized water (5 Stage Milli-Q Plus System: Millipore Corporation, Bedford, Mass.) and vortexed for 2 minutes to make fungal suspension. Sterile MEA plates were inoculated with the fungal suspension. The plates were incubated in separate chambers at 25 °C for different time periods to get fungal cultures of three different ages (old: 28~32-weeks old; mid-age: 8~9-weeks old; young: 4~5-weeks old).

Experimental set-up: The experimental facility is schematically shown in Fig. 1. After incubation, fungal propagules on agar plates were released by HEPA-filtered air at a flow-rate of 27 L/min using a newly developed Fungal Spore Source Strength Tester (FSSST), which is designed to aerosolize fungal propagules from contaminated surfaces by high speed air jets (Sivasubramani *et al.* 2003). The released fungal propagules entered in an Electrical Low-Pressure Impactor (ELPI) (TSI, Inc. St. Paul, MN) at a flow-rate of 30L/min. The ELPI is a real-time particle size analyzer consisting of an electrical aerosol charger, real-time multi-channel electrometers and a low-pressure cascade impactor classifying particles according to their aerodynamic sizes into 12 size fractions in the size range of 0.03-10 µm. The number concentration of each size fraction is measured electrically. The aerosolization part was placed inside a class II biosafety cabinet, and a HEPA-filter was connected to the exit flow from the ELPI to collect all remaining fungal propagules to prevent human exposure and contamination of the laboratory environment.

Figure 1. Experimental set-up



RESULTS

The real-time measurement by the ELPI showed bimodal size distribution of airborne fungal propagules for all the cultures of three different ages. One mode was in the size range of 1.26-1.99 μm representing intact spores, and the other was in the range of 0.07-0.31 μm representing the fragments. Minimum fungal particle concentrations observed at 0.50-0.80 μm separated the spore and fragment peaks. Thus, the particle size of 0.50 μm was selected as the lower counting limit separating spores from fungal fragments in the data analysis. The size distributions of fungal propagules for three different aged cultures are summarized in Table 1. The geometric mean concentrations of fungal fragments ranged from 850 to 1,200 particles cm^{-3} , while the concentrations of fungal spores ranged from 100 to 200 particles cm^{-3} showing much higher release of fungal fragments than spores. The release of fungal fragments was found to vary depending on the age of the fungal culture. The concentration ratio of fragments to spores was calculated for each age to normalize the amount of fragments. The results showed that the old culture released fungal fragments 1.4 times more than the young and mid-aged culture. The age of fungal culture also affected the size distribution of fungal spores showing a mean spore size of 1.84 μm for the mid-aged culture, 1.68 μm for the young culture, and 1.55 μm for the old culture. Smaller sized particles were measured for old fungal culture, which were not detected for cultures of other ages. The mean size of fungal fragments was 0.25-0.26 μm and was not different for the cultures of three different ages.

Table 1. The Size Distribution of release fungal propagules from three different aged fungal cultures.

		Fragments (0.04-0.50 μm)	Spores (0.50-8.18 μm)	F-S ratio
Young (4-5 weeks)	$(D_a)_g$ (μm)	0.25	1.68	6.21
	σ_g	1.34	1.94	
	C_g (particles cm^{-3})	1174.10	188.95	
	σ_g	3.09	204.19	
Mid-aged (8-9 weeks)	$(D_a)_g$ (μm)	0.26	1.84	6.07
	σ_g	1.34	1.98	
	C_g (particles cm^{-3})	1013.95	167.08	
	σ_g	2.47	178.27	
Old (28-32 weeks)	$(D_a)_g$ (μm)	0.26	1.55	8.50
	σ_g	1.36	1.80	
	C_g (particles cm^{-3})	846.34	99.58	
	σ_g	3.66	3.74	

$(D_a)_g$ is the geometric mean of the aerodynamic diameter; σ_g is the geometric standard deviation;

C_g is the geometric mean of the number concentration;

F-S ratio is the concentration ratio of fragments to spores.

DISCUSSION

This study confirms our previous results on the release of large quantities of fungal fragments from the agar plates. The number concentration of released fungal fragments measured by the ELPI was much higher than that of fungal spores and showed a bimodal distribution of fungal propagules. Moreover, our studies (Górny *et al.* 2002; Cho *et al.* 2002) on immunochemical analysis on fungal propagules have shown that fungal fragments contain immunologically active components, and the immunochemical activity of fragment samples was even higher than that of spore samples.

High immunological reactivity together with the large number and small particle size of released fungal fragments may contribute to the adverse health effects.

Interesting new finding in this study is that the size distribution of fungal propagules varies depending on the age of the fungal culture. It was shown that the release of fungal propagules from the old fungal culture was smaller than from the

mid-aged and the young culture. This resulted in the highest number concentration ratio of fragments to spores for the old culture. Smaller sized particles were measure for old fungal culture, which were not detected for cultures of other ages. The mid-aged culture showed the largest spore size, whereas the old culture showed the lowest fragment-spore number concentration ratio. The size of fungal fragments was found to extend as low as 0.07 μm , which makes the sampling and analysis of these particles challenging in the field conditions.

CONCLUSION AND IMPLICATIONS

This study provides detailed information on the characteristics of fungal fragments, which was not much explored previously. The confirmed existence and size distribution of fungal fragments raise the need of development of an advanced fungal sampling technique and new exposure assessment method for mold-contaminated environment.

ACKNOWLEDGMENTS

This research was partially supported from National Institute for Occupational Safety and Health (NIOSH) Education and Research Grant No. T42/CCT510420 to University of Cincinnati on Pilot Research Project.

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MICRO-PARTICLES FROM FUNGI

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ABSTRACT

At a workshop in 1999 we announced that particles with an aerodynamic diameter smaller than spore were released from *Trichoderma harzianum* cultures. Because of their size, these particles may be of interest in connection with health effects of fungi. We have studied the fungal production, origin and composition of particles smaller than spore size ($<1.3 \mu\text{m}$). These particles were called micro-particles and they were produced by eight fungal species and during growth on different materials. Some fungi released more micro-particles than spores. The micro-particles were released from fungal cultures of different ages, and the release increased with age and during autolysis. Microscopy revealed that particles of micro-particle size were released during autolysis. As micro-particles can be released before sporulation, exposure can occur where fungal growth is not yet visible. Micro-particles contained proteins and DNA or RNA. Thermal desorption of micro-particles produced volatile compound mixtures different from reported volatile metabolites. Suspensions of micro-particles were alkaline and did not contain NAGase.

INDEX TERMS: *Chaetomium globosum*, Fungal components, autolysis, Hyphal fragments, *Trichoderma harzianum*.

INTRODUCTION

At the Danish-Finnish workshop on molds in buildings in 1999 we announced that particles with an aerodynamic diameter of 0.6-0.8 μm were released when cultures of *Trichoderma harzianum* were subject to an airflow (Kildesø *et al.* 1999). These particles were smaller than spores of *T. harzianum*. The small fungal particles, which we have called micro-particles, may be of particular interest in connection with health effects caused by exposure to fungi. They are of interest because of their small aerodynamic diameters compared to spores. Micro-particles are expected to have the ability to penetrate small slits in building constructions, and to stay in the air for a longer time than spores. In addition, the large surface area relative to mass and the possible alveolar deposition may be important for possible biological

effects of the micro-particles. The constitutive of micro-particles are not known, but they are of particular interest both in relation to human exposure and in understanding the biology of fungi. Release of hyphal fragments has been reported (Gorny *et al.* 2002; Li, Kendrick 1995) to constitute 6.3% of the numbers of airborne indoor fungal spores (Li, Kendrick 1995). It could be speculated that these micro-particles also are hyphal fragments.

The aim of the study was to gain knowledge of potential exposures to micro-particles by investigating the production of fungal micro-particles in relation to different growth conditions and in relation to different fungal genera known to cause pulmonary effects. The release of micro-particles is studied before and during sporulation and during autolysis. Furthermore, we are studying the composition of the micro-particles.

METHODS

The investigations are described in Table 1 and in the following.

Fungal isolates: *Aspergillus versicolor* (Vuill.) Tiraboschi (IBT 16000), *A. ustus* (Bain.) Thom & Church (IBT 14925), *Chaetomium globosum* Kunze ex Steud. (IBT 8828), *Cladosporium sphaerospermum* (Fres.) de Vries (IBT 7710), *Penicillium chrysogenum* Thom (IBT 14920), *Pythium oligandrum* Drechsler (MM1), *Schlerotinia schlerotiorum* (Lib.) de Bary (CP1517), *Trichoderma harzianum* Rifai (IBT 9153), *Ulocladium sp.* (IBT 7155) and *Verticillium lecanii* Viégas (a gift from Dr. D. Yohalem) were used in this study.

Growth of fungi: As a standard the fungi were cultivated on sterilized wet, wall-papered gypsum boards (0.08m²) or floor pasteboards (0.018 m²) and incubated at a RH of approximately 97%. Distilled milli Q water was used for wetting the boards. Inoculation was performed by spraying a spore suspension (10⁶ spores/ml) onto the boards (1x10⁷ spores/m²) using an atomizer. The gypsum boards were placed in stainless steel boxes with tightly fitting glass covers, and a saturated solution of potassium sulfate controlled the RH (Wexler, Hasegawa 1954). Floor pasteboards were placed in glass Petri-dishes (diam. 0.15 m). The fungi were incubated at 22°C in darkness.

Fungi were cultivated on gypsum boards or floor pasteboards for 40 days to study their particle release or to study the composition of micro-particles (Table 1). *Penicillium sp* cultivated on oranges and *V. lecanii* and *C. globosum* cultivated on Potato Dextrose Agar (PDA) (Oxoid, England) for 21 days were included in the study to investigate the particle release from cultures on these media. In another experiment with *C. globosum*, the gypsum boards were soaked for 24 h in 300 ml carbonate buffer (pH 10, Radiometer) or milli Q water 40 days after inoculation. The non-absorbed liquid was removed and fungal cultures re-incubated for 3 days.

This base-treatment induces autolysis of fungi (Lahoz 1979). The experiment was also performed with 40 day old *C. globosum* cultures on floor pasteboards. In that experiment the floor pasteboards were soaked for 12 h in 20 ml of the same buffer. Particle release was measured as described below. In addition samples from the surface were taken by cutting out 40 mm diameter circular pieces of the wallpaper. The wallpaper was shaken (500 rpm, 15 min) in a sterile 0.05 % Tween 80 and 0.85 % NaCl aqueous solution and the suspension used in protein assays. The experiment was performed in triplicate. Finally, the effect of culture age of respectively *T. harzianum* and *P. chrysogenum* on micro-particle release was studied in triplicate after respectively 14, 28 and 42 days and after 5, 12, 17, 23, 28, 33 and 38 days of growth on gypsum boards.

Table 1. Performed investigations

Investigation	Included fungi	Growth medium	Methods
<u>Different fungi:</u> release of particles from different fungi	<i>A. versicolor</i> , <i>A. ustus</i> , <i>C. globosum</i> , <i>C. sphaero-spermum</i> , <i>P. chrysogenum</i> , <i>T. harzianum</i> , <i>Ulocladium sp.</i> and <i>V. lecanii</i>	Gypsum boards	APS
<u>Material:</u> release of particles from cultures on different materials	<i>A. versicolor</i> , <i>C. globosum</i> or <i>Penicillium sp.</i> , <i>V. lecanii</i>	Gypsum boards; floor pasteboards, oranges, PDA	APS
<u>Age:</u> release of particles as affected by age of cultures	<i>C. globosum</i> , <i>P. chrysogenum</i> ,	Gypsum boards	APS
<u>pH:</u> release of particles during raised pH causing autolysis	<i>C. globosum</i>	Gypsum and floor paste boards with different pH	APS
<u>Composition:</u> composition of micro-particles	<i>A. versicolor</i> and <i>C. globosum</i>	Gypsum boards; floor pasteboards	Acridine orange, protein assays, GC/MS, pH, APS
<u>Autolysis:</u> release of particles from autolysing hyphae	<i>S. sclerotiorum</i> and <i>P. oligandrum</i>	WA	Microscopy and labelling with WGA

Measurement of particle release and sampling of micro-particles: A Particle-Field and Laboratory Emission Cell (P-FLEC) (Chematec, Denmark) was used for measuring the release or re-suspension of at an adjustable airflow. Emission of particles was induced by air jets, directed to the surface of the boards in a 45° angle. The jets were generated over a surface area of 0.013 m² for a period of 60 seconds. The air velocity over the surface was 1.5 m/s. The fungal particles released from the surface were transported to an Aerodynamic Particle Sizer (APS 3320, TSI Inc., U.S.A.). Alternatively micro-particles were collected on 0.4 µm polycarbonate or GF/A filters using two Triplex cyclones (scc 1.062, BGI, INC, Waltham, MA) with a flow rate of 3.5 Lpm. Fungal spores without micro-particles were present on the inner site of the cyclones. In some experiments micro-particles were sampled on filters together with spores using IOM samplers. The APS measures total number of particles with an aerodynamic diameter (d_a) between 0.3 and 0.5µm and numbers of particles in 52 intervals with d_a between 0.5 and 20 µm. The d_a 's of most fungal spores are larger than 1.8 µm and in this study, fungal particles smaller than 1.3 µm are called micro-particles.

Release of micro-particles from filters: The polycarbonate filters were shaken (500 rpm, 15 min) in a sterile 0.05 % Tween 80 and 0.85 % NaCl aqueous. The suspensions were used immediately for cultivation analysis or frozen at - 80°C and later used for microscopy, protein assays and measurement of pH.

Protein assays and measurement of pH: Proteins and pH was measured in suspensions of micro-particles and in suspensions of fungal material released from *C. globosum* colonized wallpaper. Total protein was quantified by the DC protein assay (Bio-Rad laboratories) with Bovine serum albumin as a standard. To quantify the activities of N-acetyl-β-D-glucosaminidase (EC3.2.1.30) (designated NAGase) and β-glucosidase (EC 3.2.1.21) the release of *p*-nitrophenol from the substrates *p*-nitrophenol-N-acetyl-β-D-glucosaminide or *p*-nitrophenol-N-acetyl-β-D-glucopyranoside (Sigma Chemical Co. U.S.A.) was quantified according to Madsen and Neergaard (1999). Appropriate controls without either the enzyme or the substrate were run simultaneously. One unit of enzyme activity is defined as the amount of enzyme, which releases 1 µmol of *p*-nitrophenol ml⁻¹ enzyme min⁻¹. The measurements were performed on six micro-particle samples of each fungus.

CFU of *C. globosum* and numbers of perithecia: Ten-fold dilutions of the particles collected on the polycarbonate filters were eluted using sterile 0.05 % Tween 80 and 0.85 % NaCl aqueous solution and plated onto Dichloran Glycerol Agar (Oxoid CM729) (DG18) supplemented with penicillin (30mg/L) and streptomycin (30 mg/L) and incubated at 25°C. The number of colony forming fungal units (cfu) was counted. In the base-treatment experiment the numbers of *C. globosum* perithecia on gypsum boards or floor pasteboards were counted by stereomi-

crosscopy in 8 randomly chosen areas (38 mm²) on both treated and non-treated gypsum boards.

Microscopy of micro-particles and of autolysis

Micro-particles of *C. globosum* and *A. versicolor* were stained in 20 ppm acridine orange (Merck) in acetate buffer and observed and counted by microscopy under UV light (Orthoplan; Leitz Wetzlar). Acridine orange stains RNA and DNA (Bruno, Mayo, 1995). The numbers of micro-particles were determined until a total number of at least 400 particles were counted. The fungi *S. sclerotiorum* and *P. oligandrum* were cultivated on water agar (Madsen, Robinson, Deacon 1995) since *P. oligandrum* induces lysis of *S. sclerotiorum* hyphae (e.g. Laing, Deacon, 1991; Madsen, Neergaard 1999). This method was chosen because it is possible to observe the autolysis process. The fungi were incubated with the lectin wheat germ agglutinin (WGA, Sigma Chemical Co., St. Louis, MO, U.S.A.) (50 µg/ml) in phosphate buffer saline (PBS, pH 7.2) at room temp. in darkness. The cultures were rinsed with PBS. Fluorescence was observed by microscopy. WGA labels N-acetyl-glucosamine residues.

Collection and identification of volatile metabolites: One half of a GF/C filter (d 47 mm) containing the collected particles was placed directly in a Perkin Elmer (PE) thermal desorption tube and heated to 170°C for 20 min in a PE ATD-400 thermal desorber coupled to a Kratos Profile GC/MS system and analyzed using the standard parameters. A piece of deactivated silica capillary which was inserted through the transfer line to about 2 cm from the ATD cold trap was directly connected to a CP 19CB silica capillary column (Chrompak, 60m x 0.32 mm, 0.2µm). The split ratio at this ATD/GC interface was 1:7. Desorption conditions were 250°C for 20 min., box and transfer line temperature was 150°C and the cold trap operating temperatures were -30 and 300°C. The analysis program was 20 -220°C at 4°C/min with 13 min at 220°C. Identification was based on comparison of retention time and agreement with spectra of authentic samples. Tentative identification was based solely on agreement of mass spectra with those in the Profile library.

Statistics: Numbers of particles released from base-treated, non-treated and water-treated gypsum boards were log-normal distributed and were tested for significance at or below the 5% level using student's *t*-test. Data were then back-transformed. The numbers of *C. globosum* perithecia were lognormal distributed, the geometric mean value calculated and the numbers compared by student's *t*-test.

RESULTS

Micro-particles were produced by all the eight studied fungi as determined by the P-FLEC and APS (Table 2). The different organisms released different numbers of micro-particles per fungal colonized area. Some species released a larger total number of micro-particles than spores. Micro-particles were produced both during growth of fungi on materials relevant to indoor air environments, but also during growth on decaying oranges and PDA. From the surface of one side of a decaying orange *Penicillium sp.* spores and micro-particles were released in the numbers 3×10^6 and 5×10^4 respectively. From cultures of *C. globosum* and *V. lecanii* on PDA both spores and micro-particles were, per surface area, released in a number corresponding to 10-20% of the numbers released from paste or gypsum boards (data not shown). Micro-particles were released from *T. harzianum* cultures of different ages and most were released from the oldest cultures (Fig.1). Similarly *P. chrysogenum* released most micro-particles from the oldest cultures, however it did also release micro-particles from 5 days old cultures, which had not yet sporulated (data not presented).

Micro-particles of *C. globosum* and *A. versicolor* were not cultivable on the agar medium DG18. The enzymes NAGase and β -glucosidase, which are secreted by *C. globosum* and *A. versicolor*, could not be detected in suspensions of micro-particles from these fungi. In contrast, micro-particles contained or were associated with other proteins (Table 3). In *C. globosum* micro-particle suspensions released from an area of 1m² gypsum boards or floor pasteboards between 8 and 80 mg protein was present. The corresponding number for *A. versicolor* micro-particle suspensions was between 6 and 40 mg protein. The pH of suspensions of micro-particles from *A. versicolor* and *C. globosum* varied between 8.0 and 9.2. The pH of the elution liquid was 6.9. During 40 days of growth of *C. globosum* or *A. versicolor* the pH of the colonised wallpaper increased from 6.1 to between 7.5 and 8.9.

Table 2. Numbers of particles produced by different fungi after 40 days of growth on gypsum boards

Fungi	Size or diameter of spores* μm	Number of particles $0.3 \mu\text{m} < d_a < 0.5 \mu\text{m}^{**}$	Number of particles $0.5 \mu\text{m} < d_a < 1.3 \mu\text{m}$	Size (d_a) and number of the most abundant particle ***	
<i>A. ustus</i>	3.2-4.5	9.1×10^3	1.5×10^4	2.6 μm	2.5×10^6
<i>A. versicolor</i>	2-3	1.0×10^4	5.0×10^5	2.8 μm	4.4×10^4
<i>C. globosum</i>	8.5-11.0 x 7.0-8.5 x 6.5-7.5	1.2×10^5	1.1×10^6	2.3 μm	6.7×10^3
<i>C. sphaerospermum</i>	3-7	280	1.5×10^5	3.3 μm	1.2×10^6
<i>P. chrysogenum</i>	3.0-4.0 x 2.8-3.5	1.6×10^4	7.9×10^5	2.6 μm	6.5×10^6
<i>T. harzianum</i>	2.8-3.2 x 2.5-2.8	4.7×10^3	2.1×10^5	5.8 μm	1.4×10^4
<i>Ulocladium sp.</i>	-	170	3.5×10^3	12.9 μm	270
<i>V. lecanii</i>	2.3-10 x 1.0-2.6	170	2.7×10^3	0.7 μm	100

*According to (Domsch *et al.* 1993), **Aerodynamic diameter. *** Only particles with $d_a > 0.5 \mu\text{m}$.

Figure 1. Numbers and size distribution of *T. harzianum* particles released 14, 28 or 42 days after fungal inoculation to gypsum boards

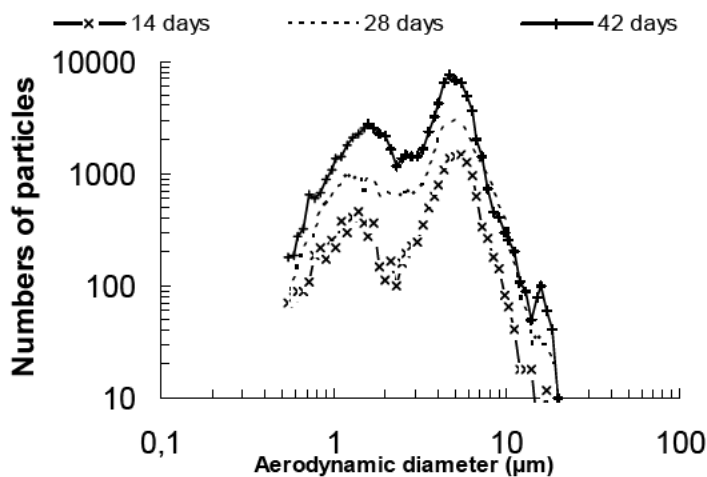
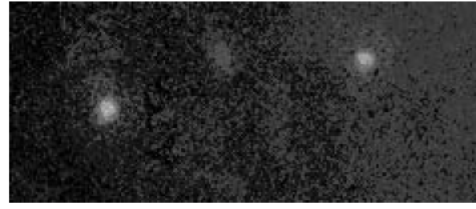


Figure 2. Two particles released from *A. versicolor* cultures on gypsum boards during exposure to an airflow. The particles are stained by acridine orange. The two particles have a diameter of approximately 0.6 μm .



When micro-particles from *C. globosum* or *A. versicolor* released by air exposure, were treated with acridine orange, particles with the sizes of micro-particles but with different shapes were observed to be fluorescent (Fig. 2). The number of micro-particles counted by microscopy after labelling with acridine orange was between 2 and 6 times larger than the number of micro-particles counted by the APS. This difference was statistically significant ($p < 0.001$). Microscopy of the lysing process of *S. sclerotiorum* hyphae reveals that particles of micro-particle sizes are released during autolysis (Fig. 3). This may be attributed to the release of hyphal contents. The outer layers of older lysed hyphae (L) are further degraded, the hyphae appear thinner and the chitin is assessable to WGA.

Figure 3. Number and size distribution of *C. globosum* particles released 40-days after fungal inoculation as affected by an air velocity on 1.5 m/s. The gypsum boards were non-treated, water-treated or base-treated.

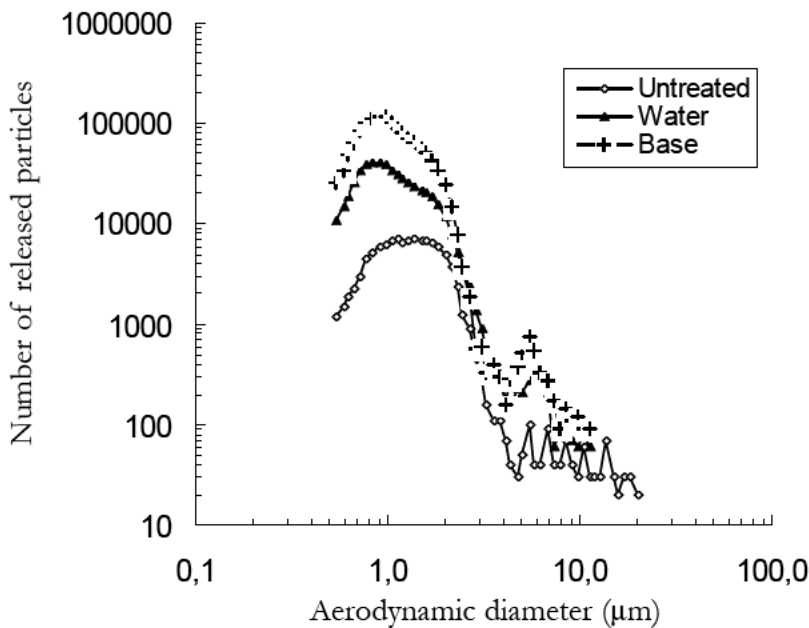


Figure 4ab: A cell in the *S. sclerotiorum* hypha (H) is lysing as a result of contact with *P. oligandrum*. The neighbour cells are vacuolated. The arrows points towards particles released during autolysis. a) Unstained, b) Labelled with WGA. The lysing cell has formed a septum-like structure (s) which is only weakly labelled by WGA. Hyphae where autolysis has occurred (L). The bar=4µm.

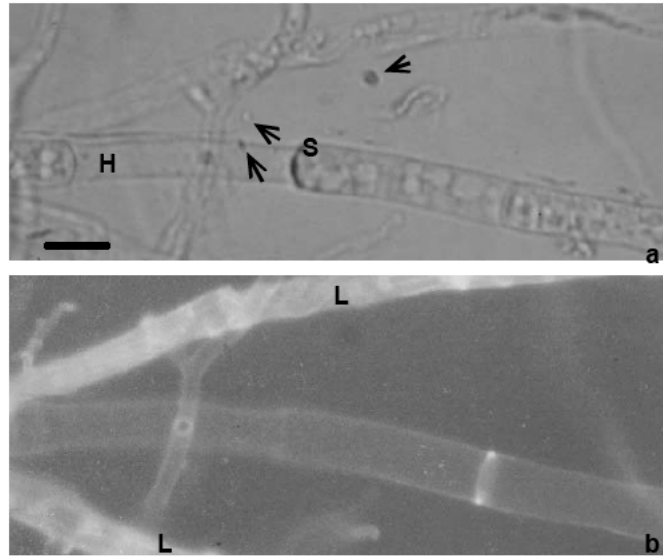


Table 3: Content of proteins in suspensions of micro-particles and in suspensions of spores and micro-particles from *C. globosum* colonised wallpaper*

Treatment	<i>C. globosum</i> micro-particles			Colonised wallpaper		
	NAGase (nmol/sek/m ²)	β-glucosidase (nmol/sek/m ²)	Protein (mg/m ²)	NAGase (nmol/sek/m ²)	β-glucosidase (nmol/sek/m ²)	Protein (mg/m ²)
None	BD ***	BD	8.0*	5.31 b	2.52 b	846.2 b
Water	BD	BD	17.5 b	10.72 a	5.10 a	15769.0 a
Base	BD	BD	34.7 a	3.43 b	1.94 c	776.9 b

*Measured after 6 weeks of growth.

**Numbers in the same column followed by the same letter are not significantly different according to student's t-test.

***Below detection level.

C. globosum released more micro-particles from cultures on base-treated gypsum or floor paste-boards than from cultures on water- and non-treated boards (Table 3; Fig. 3 a, b, c). In contrast, significantly more perithecia ($p=0.0002$) and extracellular β -glucosidase (Table 4) were present on the non-treated and the water-treated boards than on the base-treated boards. Furthermore, irregularly brown undulating hairs of *C. globosum* perithecia were more abundant on non-treated and water-treated boards than on the base-treated boards.

Table 4. Numbers of particles* released from non-treated, water-treated or base-treated gypsum boards or floor pasteboards colonised by *C. globosum*

Treatment	Gypsum boards			Floor pasteboards		
	Numbers of particles		Numbers of perithecia per mm ²	Numbers of particles		Numbers of perithecia per mm ²
	0.3 μ m < da < 0.5 μ m (gm)**	0.5 μ m < da < 1.3 μ m (gm)		0.3 μ m < da < 0.5 μ m (gm)	0.5 μ m < da < 1.3 μ m (gm)	
None	6350 a***	65120 a	0.81 a	1500 a	8300 a	0.12 a
Water	30870 b	376830 b	0.93 a	Not measured	Not measured	Not measured
Base	88770 c	511100 b	0.36 b	83000 b	510000 b	0.041 b

* Measured after 6 weeks of growth.

** Aerodynamic diameter.

*** Numbers in the same column followed by the same letter are not significantly different according to student's t-test.

The volatile compounds derived from thermal desorption of particle laden glass filters are listed table 5. Most of the volatile compounds characterized have been encountered in previous studies of other mold species from buildings (Wilkins, Larsen, Simkus, 2000). Exceptions included alkyl pyridines and cyclohexyl isocyanate. The most frequently detected compounds were ethanol, acetone, acetic acid, alkyl pyridines and 1-octen-3-ol. In *Chaetomium* micro.-I, the particles were harvested after 6 weeks compared to *Chaetomium* micro.-II and all the other experiments reported in table 5.

Table 5. Volatile compounds characterized from thermal desorption of particles from *C. globosum* and *A. versicolor* grown on gypsum board or floor pasteboards for 8 weeks.

SUBSTRATE → PARTICLES — COMPOUND ↓	<i>C. globosum</i>				<i>A. versicolor</i>			
	GYPSUM MICRO. I ^A	GYPSUM MICRO. II	PASTE. MICRO.	GYPSUM MICRO + SPORES	PASTE MICRO.	GYPSUM MICRO.	GYPSUM MICRO +SPORES	PASTE SPORES
Acetaldehyde	1,5							3
Ethanol	3	3	1	1		89	4	2
Acetone	2	40	4	4	1	13	8	2
3-buten-2-one		1,5	1					
2-butanone		2	2		1			
methyl 2-butenolate ^T				4				
1-octene artifact [?]	4							4
acetic acid	3	6		3	3	3	1	
Unknown mw 112			5					1
Cyclopentanone								0,5
Alkyl pyridines ^T	+	+	+	+	+	+	+	
Unknown mw 98				1,5				0,5
Styrene				2				1
Limonene								1
Cyclohexyl isocyanate ^T					3,5	5 ^c		1
1-octen-3-ol	4	6		1			2	
Octanol isomer				4				
Unknown								2,5

A = harvested after 6 weeks

T = tentative identification

C = cochromatography.

DISCUSSION

Micro-particles were produced during growth of the eight studied fungal species growing on building materials and *Penicillium sp.* growing on an orange; the species belonged to 7 different genera indicating that production of micro-particles may be a common phenomenon. The production of micro-particles was not related to a certain age or stage of a fungal culture as they were released during mycelial growth and during sporulation. The fact that micro-particles also were released before sporulation, suggests that exposure to micro-particles could occur before fungal growth is visible. The presence of DNA or RNA and protein indicate that the micro-particles are of fungal origin and not fragments of the growth medium. In exposure studies fungi are sometimes quantified by cultivation. The micro-particles were not cultivable on DG18. Hence, exposures to fungal micro-particles cannot be assessed by this method.

From some cultures more micro-particles were released than spores, indicating the possibility of high exposures to these particles. Theoretically, one mg of micro-particles of *C. globosum* is equivalent to about 5×10^9 micro-particles and one mg spores to about 1×10^7 spores. Thus, one mg of fungal dust can represent a very large number of fungal micro-particles.

Fungal autolysis is a process involving partial permeabilization of the cellular envelope and leakage of intracellular material causing degradation of some cell wall material (White *et al.* 2002). Results also indicate a reduction in tensile strength of hyphae (Li *et al.* 2002). Intrinsic factors as fungal aging, programmed cell death and differentiation and extrinsic factors as nutrient limitation and physical stress are effectors of autolysis (White *et al.* 2002). Because we observed, that micro-particles were released during all growth stages, but increased during aging, we studied whether the numbers of released micro-particles was increased after treatment of the colonised gypsum boards with a basic buffer that induces autolysis. As seen from the results, autolysis is associated with an increase in numbers of released micro-particles suggesting that micro-particles may be autolysis products. Part of this effect of basic-treatment may also be caused by the increase of water content of the boards. This is likely since treatment with water also caused an increased release of particles. This effect of water may be a result of an induced metabolic activity visualized by the increased β -glucosidase activity. The observed increased micro-particle release and protein secretion (including an increased NAGase secretion) may also be caused by increased hyphal differentiation or by starvation. This is likely because some proteins, including NAGase, are secreted during hyphal differentiation and starvation (Reyes, Lahoz, Cornago 1977; White *et al.* 2002). By microscopy we observed that autolysing hyphae of *S. sclerotiorum* release particles of micro-particle size. Cells which autolysed earlier had lost the outer layers of

their walls, but the chitin layer was stable compared to the cellular content. In studies of fungi in liquid cultures no cytological evidence of wall autolysis was obtained at the end of autolysis and cell walls may even remained intact after autolysis for 45 days (reviewed by Lahoz *et al.* 1986a). The enzyme NAGase, found in hyphal walls (Gooday 1995; Reyes, Lahoz, Cornago 1977; Reyes, Lahoz, Val Moreno 1979) could not be measured in suspensions of micro-particles. The stability of hyphal walls, the fact that NAGase could not be measured and the observation that micro-particles were not cultivable suggest that micro-particles are not complete fragments of hyphae (with walls) but have a different constitution.

The pH increase during fungal growth on wallpaper is in accordance with a pH increase during growth of *C. globosum* in liquid media (Kahlon, Kalra 1986). An increased pH of a growth medium is also seen during autolysis (Lahoz *et al.* 1986b; Martinez *et al.* 1986; Reyes *et al.* 1985) and is associated with diffusion of Ca^{2+} (White *et al.* 2002). Interestingly, the suspensions of micro-particles were also alkaline.

Rather small quantities of volatile metabolites were collected after thermal desorption of microparticles and spores on (in) glass fiber filters. Previously, only 2-methyl-1-propanol was detected during growth of *A. versicolor* on plasterboard (Wilkins *et al.* 2000), while α -Pinene and small amounts of 2-methyl-1-propanol, 2-pentanone, 1-hexanol and 3-octanol have been identified in volatiles from the growth of *C. globosum* on plaster board (Korpi, Pasanen, Viitanen 1999). Mixtures of volatiles in previous studies were probably dominated by volatiles derived from hypha, the dominant biomass. Alkyl pyridines have been observed previously in volatile mixtures from mold growth on nitrogen rich laboratory media (unreported observations). The formation of alkyl pyridines may explain the observed basicity of micro-particle suspensions. One striking observation was the detection of different volatile compounds from spores and mixtures of spores and micro-particles compared to pure micro-particles; methyl 2-butenate, cyclopentanone, an unidentified compound with MW 98, styrene and limonene were apparently associated with spores. The ubiquitous microbial metabolites isobutanol, and 2- or 3-methyl-1-butanol were not detected in any mixture (< 2 ng). To conclude whether these differences are representative will require much more experimental data. Some differences can be seen for volatiles derived from micro-particles of different ages.

CONCLUSION AND IMPLICATIONS

Micro-particles were produced by the studied fungi during growth on different materials. Thus production of micro-particles seems to be a common phenomenon. Some species, released more micro-particles than spores and they became easily airborne. Due to the small aerodynamic diameter of micro-particles, compared

to spores, they may be able to penetrate small slits in e.g. building constructions, and they are expected to tend to stay in the air and to exhibit alveolar deposition. Exposure may occur where fungal growth is not visible as micro-particles can be released before sporulation occurs. Therefore, micro-particles may be of particular interest in connection with health effects caused by exposure to fungi

The composition of micro-particles may be of relevance in evaluation of their health effects. We found that micro-particles contained proteins, DNA or RNA and they may contain a different pattern of volatile compounds than spores or hypha. Suspensions of micro-particles were alkaline (pH 8.0-9.2). Particles in the same size range as micro-particles were produced during autolysis in *C. globosum* cultures. It is not known whether these particles are the same as micro-particles. However, it is remarkable that a very large number of particles were produced during autolysis and that they easily become airborne when exposed to an airflow. Observations by microscopy confirmed that particles of micro-particle size can be released during autolysis. Together these results suggest further studies to determine whether the micro-particles are autolysis products and whether micro-particles can affect human health.

ACKNOWLEDGEMENTS

The Danish Working Environment Council (ASC) is acknowledged for financial support and S. H. Nielsen and Kj. Larsen for technical support.

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INDOOR BACILLI AND STREPTOMYCETES PRODUCE SUBSTANCES TOXIC TO MAMMALIAN CELLS

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ABSTRACT

Fungicidal strains of *Bacillus cereus*, *B. amyloliquefaciens*, *Bacillus sp.*, *Streptomyces anulatus* *Str. albidoflavus* and *Streptomyces sp.* were isolated from moisture damaged indoor environments. Forty % of the strains produced methanol soluble, heat stable low molecular weight (< 2 kDa) substances toxic towards human (neural, lung, HeLa) and animal cells (feline lung, boar spermatozoa). Most toxic were the extracts prepared from indoor *Str. anulatus* and *B. cereus* strains. These strains produced toxic peptides, valinomycin and cereulide, respectively, 200 to 500 ng mg⁻¹ bacteria (wet wt). Substances from 1 – 4 µg (biomass wet wt) of indoor *B. cereus* or *Str. anulatus* caused in feline lung cells (in 0.2 ml) apoptosis-like cell death and depolarised mitochondria in all test cells. Moldy building material contained valinomycin, 33 ng g⁻¹ or 9 ng cm⁻² of wall material. Indoor *Bacillus amyloliquefaciens* substance caused death of feline lung cells in 1 d by a mechanism different from cereulide or valinomycin.

INDEX TERMS: Bacterial toxins, lung cells, neural cells, mitochondrial damage, apoptosis, boar spermatozoa

INTRODUCTION

Streptomyces and *Bacillus* are frequently isolated from water damaged houses colonized with fungi (Salkinoja-Salonen *et al.* 1999). Producing antibiotic substances effective towards bacteria and fungi enables *Streptomyces* and *Bacillus* to compete with other desiccation resistant microbes. *Streptomyces* and *Bacillus* utilize complex organic molecules as chitin, the cell wall component of fungi, and latex (Jendrossek *et al.* 1997). Moldy surfaces in water damaged buildings may provide favourable growth habitats for these bacteria.

Streptomyces are known to produce toxic substances and as colonizers of moldy buildings (Andersson *et al.* 1998). Antibiotics of *Streptomyces* and *Bacillus*, toxic to eukariotic cells are known as research tools in molecular biology, over 30 of these are commercially available. But indoor *Streptomyces* and the substances they produce are not well known. *Bacillus* species were shown to produce agents toxic to mammalian cells (Andersson *et al.* 2002; Taylor *et al.* 2003) but in moldy buildings such substances have gained little attention. We describe here *Streptomyces* and *Bacillus* isolated from indoor environment producing bioactive agents inhibitory to mammalian cell functions including human.

METHODS

Isolation and toxicity testing of bacteria: Samples from indoor environment were plated on tryptic soy agar. After 14 d individuals colonies exhibiting antagonistic activity towards surrounding bacteria or fungi were harvested with a loop and suspended in methanol to the density of 25-50 $\mu\text{g wet wt } \mu\text{l}^{-1}$. The suspension was heated 10 min in boiling water. 200 μl of extended boar semen was blended with 5 μl of the heated extract, incubated at 37°C for 5 min and inspected for motility of the sperms. Nonmotile sperms were stained with JC-1 to detect changes of membrane potentials and vitality stained with Calcein-AM and propidium iodide to detect cell membrane damage (Hoornstra *et al.* 2003). Toxicity towards feline foetal lung cells (Nikulin *et al.* 1996) was tested with 10 μl of the heated extract. Human cell lines, lung (Calu-3), neurons (Paju-2) and HeLa were exposed for 20 min to the same extracts and stained as described (Jääskeläinen *et al.* 2003).

Analytical methods and chemicals: The bacterial extracts were analyzed by Liquid chromatography-ion trap mass spectrometry (LC-MS) (Hägglom *et al.* 2002). The bacteria were identified by whole cell fatty acids composition (FAME), 16 S rDNA gene sequencing and ribotyping (Andersson *et al.* 1998; Pirttijärvi *et al.* 1999). Valinomycin was purchased from Sigma (St Louis, MO). Cereulide was purified from *Bacillus cereus* F5881/94 (Jääskeläinen *et al.* 2003).

RESULTS

Occurrence of bioactive substance producing bacteria in indoor samples:

We searched from indoor environment for bacteria emitting agents toxic towards mammalian cells. The bacterial colonies were picked from culture plates of four indoor samples of different origins (air, dust, and two building materials). Isolates showing toxic response on mammalian test cells were processed to pure cultures. Eighty isolates were identified as species of *Streptomyces* and *Bacillus*.

Results in Table 1 shows the subcellular effects observed when substances that leached from the bacteria into methanol in hot water bath were tested using boar spermatozoa as test cells. From 33 colonies substances were obtained that inhibited boar sperm motility. Substances from 14 of these depolarised mitochondria and hyperpolarized the plasma membrane. The agents affecting the membrane potentials of the plasma membrane ($\Delta\Psi_p$) and the mitochondria ($\Delta\Psi_m$) in the extracts obtained from three *Bacillus cereus* strains were analyzed by LC-MS and identified as cereulide (1,15 kDa). The agents from four strains of *Streptomyces anulatus* were similarly analyzed and the effective substance identified in each of them as valinomycin (1,11 kDa). Cereulide and valinomycin are related peptides active as potassium ionophors.

Substance from *Bacillus amyloliquefaciens* strains (n = 10) depolarised mitochondria and the plasma membrane of boar sperm cells. The toxin of 1,22 kDa was detected in these extracts by LC-MS. Substances from 4 strains of *Streptomyces albidoflavus* and from one *Bacillus sp* isolate inhibited sperm motility without depolarising mitochondria. The substances from these strains contained no valinomycin or cereulide when analyzed by LC-MS and their biological effects also differed from those generated by valinomycin and cereulide (Table 1). Substances from one *Streptomyces sp* isolate inhibited sperm motility by depleting the plasma membrane integrity and substances from 3 isolates did so by depleting $\Delta\Psi_m$ with no detectable effect on $\Delta\Psi_p$.

Table 1. Bacterial isolates from indoor dust, building materials and air. The bacteria were tested for production of methanol soluble, heat stable agents inhibiting boar sperm motility, damaging the plasma membrane and depolarising mitochondria.

Test extract derived		Observed effects in boar sperm			
from Bacterial strains		cell membrane			mitochondria
Identified as species	Strains tested (n)	Permeability damage	Decrease of $\Delta\Psi_p$	Increase of $\Delta\Psi_p$	Depletion of $\Delta\Psi_m$
Strains that tested positive for sperm motility inhibition					
<i>Bacillus cereus</i>	8	-	-	+	+
<i>B. amyloliquefaciens</i>	10	-	+	-	+
<i>Bacillus sp</i>	1	-	-	-	-
<i>Streptomyces anulatus</i>	6	-	-	+	+
<i>Str. albidoflavus</i>	4	-	-	-	-
<i>Streptomyces sp.</i>	1	+	+	-	
<i>Streptomyces sp</i>	3	-	-	-	+
Strains that tested negative for sperm motility inhibition					
<i>Streptomyces sp</i>	24				
<i>Str. anulatus</i>	1				
<i>Bacillus cereus</i>	12				
<i>Bacillus sp</i>	10				

The results showed that the 33 indoor isolates of *Streptomyces* and *Bacillus* emitted substances that provoked at least six different subcellular effects in the sperm cells, indicating that six or more different bioactive substances were involved. Substances extractable into methanol from further 47 strains isolated from the same indoor materials had no effect on boar sperm motility, i.e. 40% of the strains of the fungicidal colonies produced substance (s) that inhibited boar sperm motility.

Toxicity of extracts of indoor bacteria towards lung cells: We investigated toxicity to dividing lung cells of substances produced by the fungicidal indoor bacteria. Feline foetal lung cells cultured on microtiter plates were used for the test. The

bacteria for these tests were selected from those displayed in Table 1 to represent different types of subcellular toxic effect or no effect toward boar sperm. The type strains of *Bacillus mycoides* and *Bacillus cereus* served as reference.

Results in Table 2 show that substance from the sperm toxic *B. amyloliquefaciens* strain 19b killed the lung cells within 1 d of exposure. The rapid lung cell killing effect of this extract was detected up dilutions of 1/620, corresponding to the toxicity endpoint of 8 mg of extracted bacterial biomass.

Table 2. Toxicity to growing foetal feline lung cells and boar spermatozoa of heat stable, methanol soluble substances extracted from indoor strains of *Bacillus* and *Streptomyces*.

Bacterial strain	Fetal feline lung cells*		Boar sperm**	
	Cell death after exposure of		Toxicity endpoint	
	1 d	3 – 5 d	µg extracted bacteria (wet wt) ml ⁻¹	
Strains that tested positive for sperm motility inhibition				
Cereulide producing <i>B. cereus</i> 7/ <i>pk4</i>	-	+	<4	0.4
Valinomycin producing <i>Str. anulatus</i> 6/ <i>AL</i>	-	+	<4	0.4
<i>Bacillus</i> sp. PA2/03	-	+	30	100
<i>B. amyloliquefaciens</i> 19b	+		8	8
<i>Streptomyces</i> sp 07/02	-	+	<4	<1.5
Strains that tested negative for sperm motility inhibition				
<i>Streptomyces</i> sp strain T2/02	-	+	>500	>100
<i>Streptomyces</i> sp 01 / 02	-	+	<4	>100
Indoor <i>Bacillus</i> sp strains (n=20)	-	-	>500	>100
Reference strains				
<i>Bacillus cereus</i> DSM13T	-	-	>500	>100
<i>Bacillus mycoides</i> ATCC 6441T	-	>500	>100	

The toxicity end point: cell death* was tested up to test dose corresponding to 500 µg* of extracted bacteria (wet wt) and sperm motility inhibition** to 100 mg** .

When exposed to substance prepared from the cereulide and valinomycin producing bacteria, the lung cells initially continued to grow. After exposure for 3 to 5 d apoptosis like cell death appeared at dilutions above 1/1028. Toxicity endpoint was not reached but it was below 4 µg of bacterial biomass (wet wt) . Similarly delayed toxicity was seen with substances from the sperm toxic *Streptomyces* sp 07/02, depolarising mitochondria.

The substances obtained from the sperm toxic *Bacillus* sp PA2/03 and *Streptomyces* sp 07/02 depolarised mitochondria (Table 1). The same substances were toxic to the lung cells as well, causing cell death after exposure of 3 to 5 d (Table 2). Interestingly, *Streptomyces* strain 01/02 induced apoptosis-like cell death after 3 – 5 d similar to the sperm-toxic, valinomycin producing strains of *Str. anulatus* (substance from 4 µg of bacteria exposing target cells in 0.2 ml). Since this strain was nontoxic to spermatozoa the strain must have produced an agent toxic to lung cells different from valinomycin. The 22 other bacteria found non toxic in the sperm assay, including the *Streptomyces* sp T2/02, induced no cell death even when the exposure was extended to 7 d at the highest test dose (500 µg).

Human cell toxicity of indoor bacteria: We exposed human cells to substances prepared from cereulide and valinomycin producing indoor isolates (Table 1) of *B. cereus* and *Str. anulatus*, respectively. The substances from these bacteria depolarised mitochondria in human lung (Calu-3) cells, neural (Paju-2) cells and HeLa cells at same exposure doses as in boar spermatozoa. The toxicity endpoint was achieved with the amount of substance extracted from less than 1 µg of bacterial biomass wet wt in 0.2 ml of target cell suspension. Valinomycin production of an indoor *Str. anulatus* isolate (6/AL) was measured by LC-MS and found to be 320 ng mg⁻¹ of bacterial biomass wet wt. Pure (commercially supplied) valinomycin depolarised mitochondria in all tested cells at doses of > 2 ng / 0.2 ml. Toxicity of the *Str. anulatus* 6/AL substance towards mammalian cells corresponded to its valinomycin content. Indoor *B. cereus* strains contained cereulide varying from 200 to 500 ng mg⁻¹ (wet wt). Toxicity to human neural (Paju-2) and HeLa cells of the substance obtained from these strains corresponded to the cereulide contents: mitochondrial depolarisation was visible after 10 to 20 min of exposure at ≥10 ng of cereulide ml⁻¹.

The obtained results strongly indicate that the mitochondrial damages observed in the cultured human cells in response to exposure to the substances emitted by indoor *Str. anulatus* and *B. cereus* were caused by the valinomycin and cereulide contained by these bacteria. The results also indicate that mitochondrial toxicity detected using boar spermatozoa as test cell is a relevant indicator for estimating human cell toxicity of microbial substances in indoor air.

Valinomycin may accumulate in building materials: Material from an indoor wall in a building heavily colonised by valinomycin producing *Streptomyces anulatus* was analyzed by LC-MS for valinomycin. The building wall contained 33 ng valinomycin g⁻¹ of building material, 9 ng per cm² of the wall. This shows that valinomycin producing bacteria actually grew in the building material and that valinomycin accumulated in indoor materials.

DISCUSSION AND CONCLUSION

In this study we demonstrate that it was possible to detect substances toxic to human cells by using boar spermatozoa as test cells. Toxic bacteria were conveniently isolated from indoor materials on culture plates prepared with no fungicide. When colonies that visibly antagonized fungal growth on the same plate were chosen, a high frequency (>40%) of these appeared to produce substances toxic to several kinds of mammalian cells, including human.

The new findings in this study are:

- 1) indoor *Bacillus amyloliquefaciens* produced substances extremely toxic to feline lung cells;
- 2) indoor *Bacillus cereus*, *Streptomyces anulatus* and *Streptomyces sp* produced substances which induced apoptosis-like cell death in fetal feline lung cells. This is the first time that indoor bacteria (other than *B. anthracis*) were shown to be toxic to mammalian lung cells;
- 3) agents inducing apoptosis like cell death from several indoor bacteria were identified as valinomycin, cereulide and one unknown toxin;
- 4) valinomycin was found in indoor wall.
- 5) valinomycin and cereulide depolarised mitochondria at low exposure dose in boar spermatozoa, human neural cells, feline and human lung cells and HeLa cells.

We used a combination of tests to detect mitochondrial toxicity as well as toxicity towards proliferating mammalian cells and showed that indoor isolates of *Bacillus* and *Streptomyces* produced heat stable agents of small molecular size which in small concentrations induced death of mammalian lung cells. Mitochondrial depolarisation is a known pathway leading to apoptotic cell death in mammalian cells. The results indicate that cereulide and valinomycin producing bacteria in indoor environment may represent a health hazard by causing apoptosis due to mitochondrial damage. Although our results are yet preliminary they indicate that moldy buildings represent a favourable habitat to bacteria producing substances toxic not only to fungi (molds) but also to mammalian cells, including human. The presence of

such bacteria in settled dust harvested from inhalation zone or air may be considered to represent a health hazard to the occupants of mold-affected buildings.

ACKNOWLEDGMENTS

This study was supported by the Academy of Finland, grant #53305 for the "Microbial Resources Research Unit". E. L. J expresses her gratitude to ABS Graduate School for the fellowship grant.

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Chapter 7

Assessment III – Mycology

Session chairs:

Robert Samson, Ken Dillon

FUNGAL IDENTIFICATION OF INDOOR MOLDS: CURRENT METHODS AND NEW DEVELOPMENTS

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ABSTRACT

Fungal identification of indoor molds is particularly important to evaluate the various aspects of the health hazards. The traditional methods of using morphological characters have the advantage that fungal contamination can often simply prepared and recognized, but the skill and experience is often lacking. New approaches for correct identification are discussed including new PCR methods and fingerprint methods using the specific physiological characters of each species. In addition the introduction of reference cultures and appropriate training are discussed in relation to quality assessment and management of the laboratory practice.

INDEX TERMS: Mold identification, microscopy, Biolog, PCR, reference cultures, quality assessment and management.

INTRODUCTION

For the investigation of mold problems in indoor environments it is important that the responsible molds detected should be identified to species level. Learning the identity of the species one can estimate the potential hazard caused by the presence of the species and possible toxic metabolites. Although the number of known fungal taxa is estimated about 80.000, the number occurring in indoor environments is thought to be less than 150 taxa (Samson *et al.* 2004). Identification of these indoor species however still caused many difficulties because of insufficient knowledge and training and the lack of good identifications schemes. Current morphological based methods are still in use while some new approaches are developed. The present paper presents an overview of the current and new methods.

CURRENT MORPHOLOGICAL METHODS

Identification of molds in general is still based on the microscopical examination of the conidiogenous structures produced on the substrate or on media on which pure cultures are grown. In comparison with bacteria molds need more time (for common species > 4-5 days) to develop which hamper a fast identification. However, molds grown on surfaces and material most often produce their characteristic structures, allowing fast recognition when using for example cello tape preparation and a simple microscope.

On the current recommended isolation media (Samson *et al.* 2004; Samson, Houbraeken, 2001) most species show their typical conidiogenous structures and with some training and experience they can be recognized directly using a dissecting and compound microscope. With sufficient experience of the indoor flora the strains do not need to be transferred to special media for identification which can save time and material.

BIOCHEMICAL CHARACTERIZATION

Identification of fungi is still primarily based on their morphological appearance, i.e. their phenotypic characters. In yeasts and bacteria, biochemical and physiological profiles are used and this facilitates the use of identification kits such API and Biolog. Chemotaxonomic approaches such as profiles of secondary metabolites (including volatiles and mycotoxins) have been proposed for identification but have not yet gained any practical significance.

The Biolog FF Micro plate performs 95 discrete tests simultaneously and gives a characteristic fingerprint pattern. Currently the FF database contains over 500 fungal taxa of indoor environments; food etc. distributed over 120 genera. Although the procedure is fast and simple it is important when the micro plates are read with a reader supplied by Biolog. Most fungal species need at least three days to respond to the specific reagents. This can vary for each strain and therefore it is crucial that the isolates should also check microscopically with a photo gallery included in the software. A better approach is that the user receives training how to isolate, detect and recognize the most common fungi. Short training courses which deal with these microscopical examination are therefore useful to follow and they have been given in various places.

MOLECULAR APPROACHES

Because of the difficulties for persons with little or no mycological knowledge it is logical that other methods are searched. New and more accurate identification methods have therefore been under development. The methods currently devel-

oped are based on PCR methods (Haugland *et al.* 1999, 2002). One of the approaches that the target DNA from building material can be used as a template for a PCR reaction using universal fungal primers to amplify the ITS area of ribosomal DNA: In the case of Quantitative PCR, quantitative data on the presence of viable and dead molds can be obtained which is not possible with the present culturable methods (Haugland *et al.* 1999).

Fungal taxonomy is continuously in a state of flux and with the molecular methods our knowledge of species delimitation is regularly improving. In many cases old taxonomies only based on morphology will become unreliable. In *Fusarium* where species identification is difficult based on characters of cultural and conidogenesis it becomes clear that the genus may exist of numerous taxa which can only be recognized by molecular methods. Also in the common indoor fungi this is the case although morphological characterization is often appropriate. For example we have recently demonstrated that *Aspergillus ustus* is not common in indoor environment but *Aspergillus insuetus* an older synonym. A polyphasic approach has shown clear differences between these two species. Also in subgenus *Penicillium* which contains several species commonly occurring in indoor environments and which all have specific ecological and mycotoxins profiles a new taxonomic scheme will be introduced with new species delimitations.

With respect to all these new taxonomic developments it is therefore important to understand that the currently available data bases are insufficient to serve for molecular methods and that it is crucial that these databases should be expanded with well identified strains which also should be publicly available. The regular discovery of new taxa in mycology also contribute to the completeness of the data bases

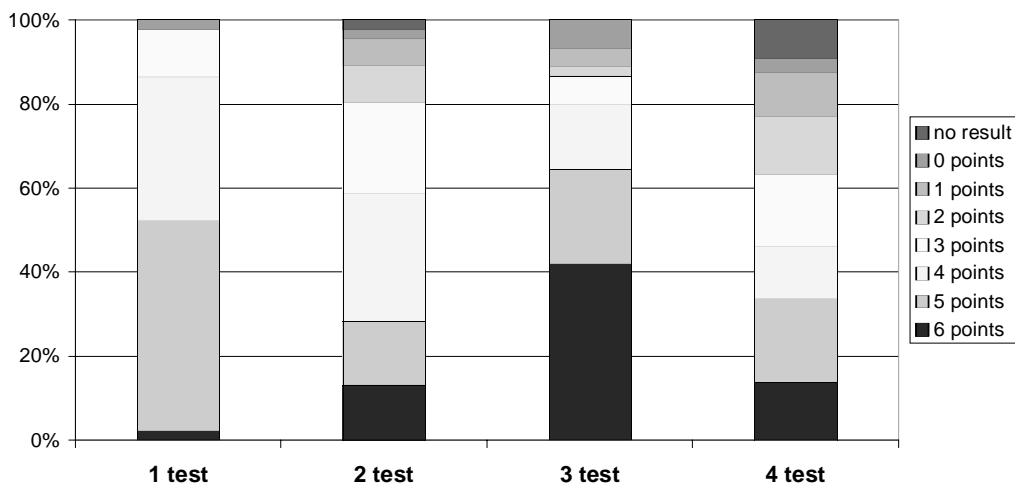
QUALITY ASSESSMENT AND MANAGEMENT

As stated above the identification for investigators with little or no skill in mycology is difficult. However, in short courses the most common species can be investigated and instructions for identification can be learned. A text book with numerous illustrations and keys to species has been published by Samson *et al.* (2004). For a quality assessment for investigating mold problems reference cultures can be valuable. In cooperation with the Federal Environmental Agency the Baden Württemberg State Health Office (LGA) the Centraalbureau voor Schimmelcultures has so far carried out four inter-laboratory tests for the 'Identification of Indoor Fungi.' (Gabrio *et al.* 2003 a, b) For each test, six strains of fungi relevant to indoor environments were sent to the participating laboratories. Prior to the test the pure cultures were checked for purity and expression of typical morphological characteristics by eight reference laboratories. The prerequi-

site to use a strain in the test was that all reference laboratories had correctly identified the species. An internal quality control measure to guarantee the purity and identity of the strains has proven to be very important before dispatching the cultures. The participating laboratories must provide a signed certificate assuring that they have done the inter-laboratory test autonomously, without assistance of other laboratories, institutes or external staff. To take part successfully, the participants had to identify correctly 4 out of 6 strains up to the species level. In the first inter-laboratory test 44 laboratories participated, 86,4% met the requirements; in the second test 46 labs took part, 58,7% met the requirements; in the third test there were 45 participants and 80% met the requirements; and in the fourth test 65 labs took part, 46,2% met the requirements (Fig. 1).

Figure 1. Results of 4 inter laboratory test in 2003 and the scoring of the participants

Inter laboratory Test 1-4 successful participation expressed as percentage



Generally the most difficult genera of fungi for identification were *Penicillium* species e.g. *P. digitatum*, *P. expansum* and *P. olsonii*. In total, 109 different laboratories participated, mainly from Germany, but also from the Netherlands, Austria, Portugal and Sweden. Inter-laboratory tests increase the quality of the analysis in the participating laboratories. This was already evident from the first few tests for identification of indoor fungi. The statistical evaluation showed that laboratories which took part more than once in the tests were more successful than new comers

The inter laboratory test are planned every 6 months and the cultures used as references will be deposited at and available from the Centraalbureau voor Schimmelcultures in Utrecht, The Netherlands (www.cbs.knaw.nl).

Besides the reference cultures also dust and air samples have been distributed among the participating laboratories. The results of the analysis are discussed and evaluated during the short courses. These analysis have been appreciated by the participating laboratories to test their skills. It is planned to expand the inter-laboratory tests to other countries and contacts to national reference laboratories are sought.

INTERNATIONAL COMMISSION OF INDOOR FUNGI

At the general assembly of the Mycology Division at the IUMS congress in Paris in 2002 it has been decided to establish an international commission dealing with and coordinating of various aspects of indoor fungi. The standardization of methods and quality assurances for laboratories active in microbial surveys and the international inter laboratory tests are primary aims of the commission.

ACKNOWLEDGEMENTS

The author is grateful to the participants of the German reference laboratories and in particularly Dr Thomas Gabrio (Stuttgart) for their cooperation and making the data available.

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GYPSUM IN NUTRIENT MEDIUM ENHANCES GROWTH OF *STACHYBOTRYS CHARTARUM*

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ABSTRACT

Seven *Stachybotrys chartarum* isolates were cultured on plain 2% malt extract agar (MEA, control), and MEA with 0.5% (w/v) pure gypsum (CaSO₄·2H₂O; GMEA). From isolates used, five originated from Finnish water-damaged buildings, one from outdoor air and one from litter (CBS 492.96). All isolates grew densely and sporulated well on both control and test media. The gypsum-amended medium, GMEA, was found to enhance *S. chartarum* growth considerably compared to MEA: the difference in colony diameter was significant from the first measurement, 2 days after inoculation. The isolates originating from concrete substrates and growing most slowly on MEA gained most from the gypsum amendment. The GMEA medium introduced might be useful for detection of slow-growing *S. chartarum*, often overgrown by other mold species on other nutrient media.

INDEX TERMS: *Stachybotrys chartarum*, growth conditions, media, gypsum

INTRODUCTION

The genus *Stachybotrys* and especially the species *S. chartarum* (Ehrenberg ex Link) Hughes (= *S. atra* Corda) is frequently found on moist cellulose materials in buildings, but also on calciferous substrates such as plaster (e.g. Gravesen *et al.* 1999; Hyvärinen *et al.* 2002). It is typically found on gypsum board, which consists of a gypsum layer between cellulose cardboard liners.

Stachybotrys chartarum is known to produce mycotoxins and it has been linked with serious diseases, such as stachybotryotoxicosis in both animals and man, and idiopathic pulmonary haemosiderosis in infants. Due to the increased health risk, the detection and identification of *Stachybotrys* has been considered important for instance in governmental remediation instructions (Morey, 2001).

Confirmation of *S. chartarum* in air or material samples by a culture method is often problematic. The slimy-sheathed spores do not easily become airborne, and up to

90% of airborne spores may not be viable (Miller 1992). Though *S. chartarum* grows effectively on certain building materials, it grows poorly and/or does not produce spores on the nutrient media as malt extract agar or DG18, often recommended for the detection of indoor fungi (Miller, 2001; Andersen, Nissen, 2000). Furthermore, it is often overgrown by other molds such as *Penicillia*, *Trichoderma*, *Mucor* and *Rhizopus*. Improvement of detection media for *S. chartarum* is therefore urgent.

Cellulolytic characteristic of *Stachybotrys* is often emphasised in a search for good detection media. However, mineral additives may act significantly on *Stachybotrys* growth. Clay minerals, especially montmorillonite have been shown to accelerate growth of *Stachybotrys* (Jong, Davis 1976). In a previous study on the fungal solubilisation of calcareous building materials, 0.5% (w/v) powdered gypsum board and other calciferous amendments (concrete, stone wool) in MEA nutrient agar were observed to enhance the growth rate of *S. chartarum* isolates (Pessi, A-M., unpublished).

Prevalence of *Stachybotrys* on gypsum substrates in buildings and acceleration of growth rate by calciferous materials lead us to examine effect of pure gypsum amendment in MEA on the growth rate of seven *S. chartarum* isolates.

METHODS

Isolates, media, and experimental set-up: The growth of six Finnish *S. chartarum* isolates and one CBS strain 492.96 (Table 1) was measured on three different media. The Finnish strains were isolated from moistened building materials and from indoor and outdoor air. Pure gypsum, calcium sulfate-2-hydrate, was provided by Riedel-de Haën®. Gypsum suspension was prepared in a small volume of sterile deionized water and added to the melt (57° C) medium after steam-sterilisation. The final compositions of the media were as follows: 20 g malt extract, 15 g agar in 1000 ml deionized water (MEA), and with 5.0 g CaSO₄ · 2H₂O (GMEA). 0.01 % (w/v) chloramphenicol was added as antibiotic. The pH of the medium surface was determined (pH-meter Russel Ltd model RL 100 and surface electrode Type KDCEF 11) and the pH of the modified medium was found to be the same as in MEA, pH 6.3. GMEA remained slightly dim; in other words, gypsum did not dissolve totally into the medium but the blend was approximately homogenous.

Table 1. Details of *Stachybotrys chartarum* isolates used

Reference number	Origin	Source, substrate
D210	Finland	indoors, airsample
D212	Finland	outdoors, airsample
D213	Finland	wall, gypsum board
D303	Finland	floor, concrete
D304	Finland	floor, concrete
D305	Finland	floor, concrete
CBS 492.96	Cuba	leaf litter

The isolates were first cultured on MEA for two weeks and suspended in dilution water (Pasanen *et al.* 1992) by flooding the agar plate. The dilute spore suspension was spread on a thin (1.5 mm) layer of MEA, which resulted in small colonies after three days. The colonies were cut with a borer into 5.0 mm discs and transferred onto the test medium, one disc per plate. All inoculations were done the same day in randomised order. Six replicates were used for each isolate and medium. The cultures were incubated at 25°C and in the dark. In order to quantify mycelial growth rate, two colony diameters perpendicular to each other were measured at 2, 4 and 7 days, and the means were calculated. Only colony diameter was measured, because it was earlier shown that colony diameter of *S. chartarum* colony is in relation to both wet and dry weight (Pessi, unpublished).

Study design and statistics: The study design formed a hierarchical structure, with medium, day and isolate as fixed effects. Each inoculate (6/isolate) was subject to three repeated measurements (day). The growth rates of different isolates were estimated for each isolate separately, using time (day) as covariate random effect.

The analyses were performed with the procedure MIXED in SAS® statistical software (version 8.2, SAS Institute Inc., Cary, N. C.). The Kenward-Rogers approximation was used to estimate degrees of freedom, and assumptions of normality were checked from the residuals.

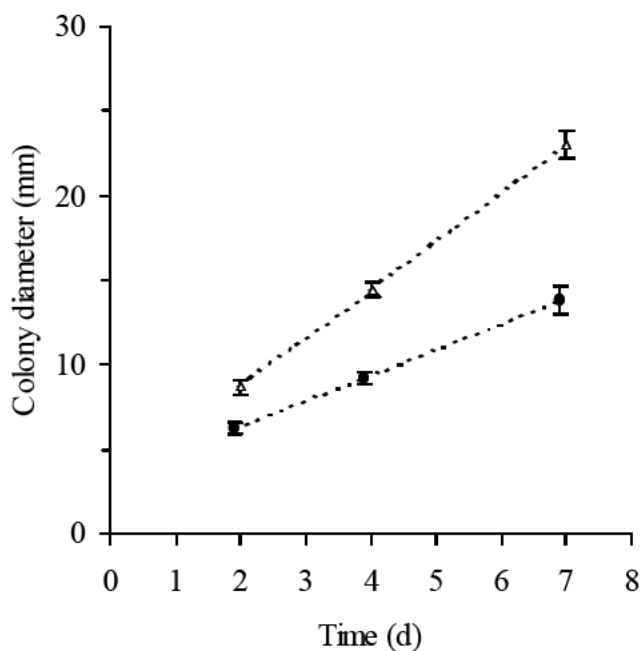
RESULTS

All *S. chartarum* isolates grew densely and sporulated on both media. GMEA was found to enhance *S. chartarum* growth considerably in comparison to MEA (Figure 1). From the beginning (2nd day after inoculation), the difference in colony diameter GMEA and MEA, 2.5 mm, was significant (95% confidence interval, C.I.= 1.9

- 3.0, Df=14.2, t=10.06, P=<0.001). After 7 days incubation, the difference had increased to 9.2 mm (C.I.=8.0 - 10.4, Df=34.5, t=16.01, P=<0.001).

GMEA was found superior for all isolates, despite the variation in their growth rates. The ratio between colony growth rates on GMEA and on MEA varied from 1.6 (isolate D210) to 3.14 (isolate D303). The slowest-growing isolates, originating from concrete substrates, enhanced their growth most. (Figure 2)

Figure 1. Colony diameter of seven *Stachybotrys chartarum* isolates on MEA (●) and GMEA (Δ).

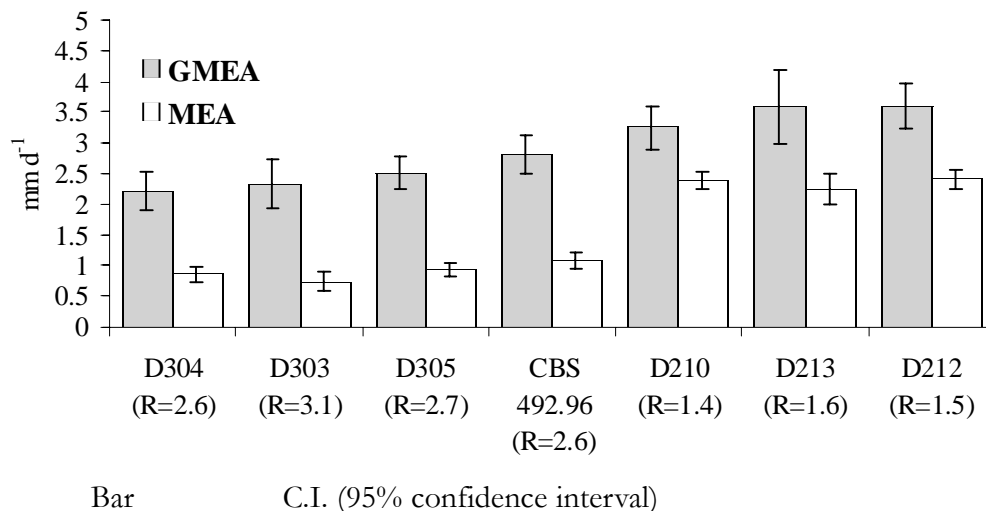


Bar C.I. (95% confidence interval)
 Time Days from inoculation by micro-colony on 5 mm ø agar disc.

DISCUSSION

Gypsum amendment in MEA medium was found to enhance *S. chartarum* growth considerably. This is in line with the study of Murtoniemi *et al.* (2002), who cultured the species on gypsum board core and cardboard liner separately. They found that wetted core supported faster and more pronounced growth than liners without gypsum.

Figure 2. Growth rate (colony diameter, mm d⁻¹) of each *Stachybotrys chartarum* isolate, and ratio (R) between colony growth rates on GMEA and MEA.



Earlier studies on media for the detection of *Stachybotrys* and the related genus *Memmoniella* have emphasised cellulolytic characteristics. Media containing cellulose or starch, including sterile filter paper on medium, medium containing wallpaper paste (Hoekstra, Samson, Summerbell, 2000), oat meal, and corn meal agar (Domsch, Gams, Anderson, 1993), have been recommended for the detection of this cellulolytic fungus. Andersen & Nissen (2000) found oat, corn and rice meal agar unsuitable for the detection of *Stachybotrys*. Even though the diameter of the colonies increased, colony density remained so low that it could be overgrown by competing fungi and/or overlooked by the inspector. Media with vegetable-based carbon sources, such as potato-sucrose agar and V8 juice agar, yielded denser colonies and were recommended in this study. However, they found no optimal medium for *S. chartarum*. In our tests, *S. chartarum* produced dense, well-sporulating colonies on GMEA.

In this study, all studied isolates sporulated on MEA. In contrast, Andersen & Nissen (2000) found that MEA supported growth but not consistent sporulation on some Danish isolates. Both sporulation and growth rate might be an isolate-specific feature; the applicability of GMEA should therefore be studied with a larger selection of isolates from broad geographical area.

GMEA may also enhance growth of other fungi. In the papers of Gharieb and others (1998; 1999), 0.5% gypsum in nutrient medium was found to slightly enhance the growth of some cellulolytic species and/or organic acid producers (*Aspergillus niger*, *Phanaerochaete chrysosporium*, *Penicillium bilaii*, *P. simplicissimum*). In

other cases, growth was reduced (*Coriulus versicolor*, *Paxillus involutus*, *Serpula himantoides*). However, in these two studies the ratio between growth rates on gypsum-amended and gypsum-free agars varied from 0.78-1.34 — less than on any *S. chartarum* isolate studied here.

CONCLUSION AND IMPLICATIONS

The new medium, GMEA, enhances *S. chartarum* growth considerably and might be useful tool for detection of the fungus. However, its applicability to air and material samples with varying spore types should be investigated further. In addition, the selection ability of GMEA for and against other molds, frequent in indoor environments, should be tested.

ACKNOWLEDGEMENTS

We are grateful to Ms. Silja Lehtimäki for technical assistance, Dr. Mika Kurkilahti for help with the statistical analyses, and Dr. Ellen Valle for checking the English language.

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NUTRIENT AND pH DEMANDS OF STREPTOMYCETES ISOLATED FROM MOLDY INDOOR ENVIRONMENTS

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ABSTRACT

The growth and the sporulation of ten streptomycete strains, isolated from moldy buildings, were studied on seven growth media in a pH range from 4.0 to 11.5 at intervals of 1.5. The pH ranges for both the growth and the sporulation were broad, i.e. minimum pH was between 4.0 and 7.0 and the maximum between 8.5 and 11.5. If the strain grew or sporulated at pH 4.0, it invariably grew or sporulated over the whole pH range studied. Although the pH ranges were strain specific characteristics, they depended also on the composition of the medium. One of the strains was studied further to evaluate the effect of the medium pH on cytotoxicity of the spores by using mouse macrophages (RAW 264.7). These results show clearly that culturing streptomycete spores at different pHs induced significant differences in their ability to evoke cytotoxicity in mammalian macrophages.

INDEX TERMS: Nutrients, pH, streptomycetes, cytotoxicity

INTRODUCTION

The gram-positive bacteria, streptomycetes, which belong to the large group of actinobacteria, are widely distributed existing in a variety of habitats, but when they are present in buildings, their existence may be indicator of moisture problems (Nevalainen *et al.* 1991). Moreover, in moisture damaged buildings, these bacteria are also able to produce toxins (Andersson *et al.* 1998). *In vitro*, the spores of streptomycetes evoke cytotoxicity and induce the production of inflammatory mediators in immunological cells (Hirvonen *et al.* 1997a, 1997b; Huttunen *et al.* 2003).

These biological effects may represent important mechanisms in the respiratory symptoms experienced by inhabitants of moldy buildings.

The nutrient composition and the pH of the environment can greatly affect the growth, sporulation, morphology, and production of secondary metabolites by streptomycetes. Streptomycetes can degrade complex polysaccharides (starch, pectin chitin), proteins (keratin, elastine) and aromatic compounds. If the amount of nutrients essential for the growth is limited, the pH of the medium becomes an important factor controlling growth. Generally, both acidophilic and alcalophilic streptomycetes have been found, but most species prefer a neutral to alkaline environment with the pH optimum lying between 6.5 and 8.0 (Kutzner 1986). The nutrient composition and the pH of the medium can influence the metabolism of streptomycetes, their growth and their biosynthesis of secondary metabolites. In this study, the interaction of the nutrients and pH on the growth, sporulation and cytotoxicity of streptomycetes isolated from moldy indoor environments was analyzed.

METHODS

Strains and media: Ten *Streptomyces* strains VTT E-99-1326 to E-99-1335 (VTT, Technical Research Centre of Finland, Biotechnology and Food Research) used in the study were isolated from water-damaged buildings (Suutari *et al.* 2002a; Nevalainen *et al.* 1991). Strains VTT E-99-1326 and VTT E-99-1331 were identified as *Streptomyces californicus* and *Streptomyces anulatus*, respectively, at the German Collection of Microorganisms and Cell Cultures (DSMZ). The strains were cultivated on seven different media (Table 1) solidified with 1.5 % agar. These media favored the growth of streptomycetes in a cultivation experiment carried out previously (Suutari *et al.* 2002b). Four of the media used in this study are commonly used for the isolation of streptomycetes: medium 1, starch-casein-KNO₃ agar; medium 2, tryptone- yeast extract-glucose agar (TYG); medium 6, glycerol-arginine agar; medium 7, tryptone-soy agar (TSA). The other three media were combinations of the media used in literature.

Table 1. The media compositions

Medium (g/l)	1	2	3	4	5	6	7
Starch	10.0						
Glucose		1.0	1.0				
Glycerol					12.5	12.5	
Na-propionate				4.0			
Soy							5.0
Casein	0.3						
Tryptone		5.0	5.0				15.0
Arginine					1.0	1.0	
KNO ₃	2.0						
NH ₄ NO ₃				4.0			
Yeast extract		2.5		2.5	2.5		
NaCl	2.0				1.0	1.0	5.0
K ₂ HPO ₄	2.0		1.0			1.0	
CaCO ₃	0.02						
Mg- and Fe-sulfates	0.05 - 0.01		0.5 - 0.01			0.5 - 0.01	
Cu-, Zn- and Mn-sulfates			0.001			0.001	

The growth and the sporulation were studied in a pH range from 4.0 to 11.5 at intervals of 1.5. The pH was adjusted with 1 M NaOH or HCl before autoclaving at 121 °C for 15 min. After autoclaving, the pH was checked from a small amount of solidified agar and readjusted if necessary. After sterilization, the pH level 11.5 was adjusted from the pH value of 10.0.

Cultivation: High density spore suspensions ($>5 \times 10^7$ ml⁻¹, 5 µl) of ten streptomycete strains were inoculated on seven media in petri dishes. The plates were incubated at a temperature of 20±2°C. The growth and the sporulation were recorded after 16 days using visual assessment and presented as 0, 20, 40, 60, 80 and 100 % of mycelium/spores from the maximum obtained for each strain. Microscopy (Labophot-2, Nikon, Tokyo, Japan) was used when the mycelium/sporulation was barely visible.

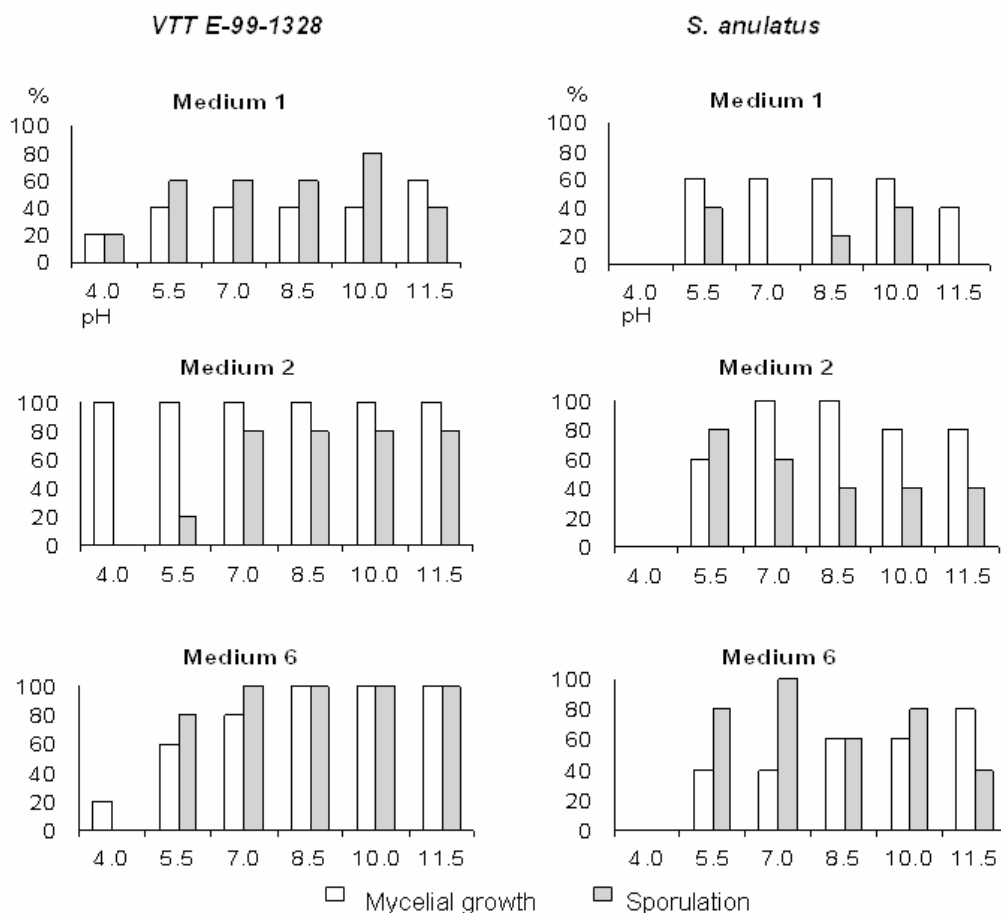
Cytotoxicity: For cytotoxicity analysis, RAW264.7 macrophages were exposed for 24 h to different doses of *S. anulatus* spores grown on medium 3. The cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) test (Hirvonen *et al.* 1997c). The data were analyzed using one-way analysis of variance and Duncan's Multiple Range test, Wilcoxon Signed Rank Test and Wilcoxon Rank Sum Test ($p < 0.05$).

RESULTS

Growth and sporulation: Figure 1 illustrates the effect of pH on the growth and the sporulation of two streptomycete strains on three media commonly used for the isolation of streptomycetes. Under identical circumstances, pH modified the growth and the sporulation, but separate strains reacted differently. In general, the growth pH ranges for the streptomycete strains studied were broad, with pH minimum ranging between 4.0 and 7.0 and pH maximum between 8.5 and 11.5. If the strain grew or sporulated at pH 4.0, it invariably grew or sporulated over the whole pH range, up to pH 11.5. Although the pH ranges were a strain specific characteristic, they depended also on the medium composition. Some of the strains sporulated only weakly under acidic conditions. With respect to the growth of the mycelium, the pH range was broadest on media 2, 3, 5 and 7; i.e. those with TYG, the modified glycerol-arginine agar and TSA. On media 4 and 6; i.e. the modified actinomycetes isolation agar and on glycerol-arginine agar, growth was optimal in the basic pH range. In contrast, on medium 1, i.e. starch-casein-KNO₃ agar, the growth was optimal in the acidic pH range.

Cytotoxicity: The spores of *S. anulatus* grown at different pHs revealed significant differences in their ability to evoke cell death in macrophages. The spores of this microbe grown at pH 4.0 were most cytotoxic (viability 6.0 %) whereas spores grown at pH 10.0 decreased the cell viability only to 58.5 %.

Figure 1. The effect of pH on the mycelium growth and the sporulation of the strain VTT E-99-1328 and *Streptomyces anulatus* on starch-casein-KNO₃ agar (medium 1), TYG (medium 2) and glycerol-arginine agar (medium 6).



DISCUSSION

Generally, we observed broad growth pH ranges for the streptomycetes strains studied. Changing the mineral solution of the glycerol-arginine agar to yeast extract increased the growth and broadened the pH range of the growth to the acidic direction. When the medium contained starch and casein, four strains even grew at pH 4.0. The amino acid metabolism may affect the growth pH range in acidic conditions. If the medium contained arginine, streptomycetes grew weakly below pH 7.0. The maximum growth at pH 5.5 was noted in the presence of protein hydrolysate tryptone.

Some of the strains sporulated less under acidic conditions, which may be due to the growing conditions or due to genetic reasons (Hopwood 1988; Chater 1989).

Our earlier studies have shown that the cytotoxicity of *S. anulatus* spores depends on the growth media (Hirvonen *et al.* 2001). Moreover, also the pH of the growth medium influenced the cytotoxicity of the spores of the same strain. This may be an important aspect in understanding the causal relationships between microbes in moldy buildings and their effects on health.

CONCLUSION AND IMPLICATIONS

The streptomycetes studied could not be divided into acidophilic or alkalophilic strains. Our results on streptomycetes isolated from indoor environments demonstrate that their pH tolerance extends over a wide range and depends on the nutritional status of the environment. The pH of the medium also modifies the cytotoxicity of the spores.

ACKNOWLEDGEMENTS

Ms Ulla Kukkonen, the late Ms Tuula Wallenius and Ms Heli Martikainen are acknowledged for their excellent technical assistance. This study was supported by the Academy of Finland and the Finnish Work Environment Fund.

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THE INTERACTIONS BETWEEN *STACHYBOTRYS CHARTARUM* AND *STREPTOMYCES CALIFORNICUS*

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ABSTRACT

Indoor bioaerosols associated with microbial growth including both bacteria and fungi in structures of buildings are a potential cause of inflammatory related symptoms among the occupants. These microbes may be enhancing or counteracting each others' effects. It has been shown that the presence of *Streptomyces californicus* and *Stachybotrys chartarum* indicates moisture and mold damages in a building. In this study we identified interactions between the bacteria and fungi on physiological state and biomass of the microbes. This was estimated on the basis of total content of fatty acid. The current experimental setting revealed that there are differences between *S. chartarum* fatty acid profile when cultured together with *S. californicus* or separately. Also the total microbial biomass of this microbe is greater when cultured together with *S. californicus* compared to separately cultured combination. These results indicate that co-cultivation affects the physiological state and the biomass of the microbes.

INDEX TERMS: Microbial interaction, fatty acid, biomass, physiological state

INTRODUCTION

Interactions between different exposures in a moisture damaged house are inevitable, because it is always a habitat of more than one microbial species (Hyvärinen *et al.* 2002). These microbes may enhance or counteract each others' effects. However, little is known about the interactions between different microbes,

although the cytotoxicity and inflammatory potential of certain separate microbes has been reported (Huttunen *et al.* 2003).

Fatty acids can be used as biomass marker for microbes. Earlier we have used fatty acid methyl esters to study the effects of plasterboard composition on *Streptomyces californicus* growth (Murtoniemi *et al.* 2003). On the other hand, differences in fatty acid composition can reveal changes in physiological state of pure culture microbes.

The gram-positive bacterial strain *Streptomyces californicus* and the fungal strain *Stachybotrys chartarum* were selected in the study based on their characteristic occurrence in water damaged buildings (Samson *et al.* 1994). We have demonstrated earlier that *Str. californicus* is highly potent to trigger the production of important inflammatory mediators i.e. NO and cytokines and to cause cell death in macrophages (Huttunen *et al.* 2003). *S. chartarum* is a well-known producer of potent mycotoxins (Jarvis, 2002).

Our recent results indicate that co-exposure to spores of bacteria and fungi i.e. *Str. californicus* and *S. chartarum* increases the inflammatory potential of these microbes in mouse macrophages (Huttunen *et al.* 2004). The co-exposure to *S. chartarum* and *Str. californicus* caused a synergistic increase in the production of IL-6. In this study we investigated more detailed interactions between these two microbes. The aim of the present study was to identify differences in microbial biomass and physiological state when the microbes are cultured separately and together on the same culture medium.

METHODS

S. chartarum was co-cultivated with *Str. californicus* on the same 2 % malt extract agar plate (MEA) inoculated in the proportion of 1:100 (*S. chartarum*: *Str. californicus*). The plates were incubated at 25°C in the dark until the microbes sporulated (17-40 days). Both strains were also cultivated separately on the same media. MEA were selected for growth media, because both bacteria and fungi were able to grow on that, although growth conditions were not optimal for bacteria. After incubation, the spores were collected with sterile loop and suspended into Hank's Balanced Salt Solution (HBSS). The spore concentration was counted using an epifluorescence microscope. Separately cultivated spores of *S. chartarum* and *Str. californicus* were combined at the same proportion as those cultured together (proportion was 1:5).

Fatty acid methyl esters: The total content of fatty acid methyl esters (FAMES) of *S. chartarum* and *Str. californicus* was analyzed according to the method by Suutari *et al.* (1990). HBSS without microbial growth were used as references and analyzed

identically with samples. The samples were stored (-20°C) and analyzed under a nitrogen atmosphere. For the quantification of FAMES, tridecanoic and nonadecanoic acid methyl esters were added as internal standards. The lyophilized samples were saponified, methylated and extracted as methyl esters. FAMES were analyzed with a Hewlett-Packard (Palo Alto, California, U.S.A.) model G1800A gas chromatograph equipped with a mass selective detector (GC-MS) using total ion monitoring. In addition, FAMES for *Str. californicus* were also analyzed using selected ion monitoring (Keinänen *et al.* 2002). FAMES are designated as the total number of carbon atoms: the number of double bonds. The prefixes *i*- and *a*- indicate *iso*-branched and *anteiso*-branched, respectively.

RESULTS

In fungi *S. chartarum*, grown separately, nine FAMES, namely straight-chain saturated 14:0, 15:0, 16:0, 17:0, 18:0, straight-chain monounsaturated 16:1, 18:1, and straight-chain polyunsaturated 18:2a, 18:2b were detected. 16:0, 18:1 and 18:2a dominated the profile with proportion of 97.8%. In the bacterium *Str. californicus*, grown separately, 16 FAMES (*i*-14:0, 14:0, *i*-15:0, *a*-15:0, 15:0, *i*-16:1, *i*-16:0, 16:1, 16:0, *i*-17:1, *a*-17:1, *i*-17:0, *a*-17:0, 17:1, 17:0, 18:1) were detected. The most abundant *a*-15:0 and *i*-16:0 accounted for 14.3 % and 46.5 % of total FAMES, respectively.

Most of the FAMES content was from *S. chartarum*. The amount of FAMES of *S. chartarum* was $349 \pm 18 \mu\text{g ml}^{-1}$ (n=5) in separately grown samples and $628 \pm 32 \mu\text{g ml}^{-1}$ (n=5) when microbes were grown together. The amount of FAMES of *Str. californicus* was $0.9 \pm 0.1 \mu\text{g ml}^{-1}$ (n=5) in separately grown samples and $2.1 \pm 0.4 \mu\text{g ml}^{-1}$ (n=5) when microbes were grown together.

The *S. chartarum* contained more 16:0 and 18:0 and less 18:1 fatty acids when grown together with *Str. californicus* than when it was grown separately.

DISCUSSION

The current data show that the FAME profiles are different for the fungal strain *S. chartarum* and the bacterial strain *Str. californicus* which enables their biomass determination while cultured both separately and together on the same culture plate. These results on *S. chartarum* are in line with the earlier report by Bishy *et al.* (1981) demonstrating that seven fatty acids, *br*-14:0, 14:0, 16:0, 18:0, 18:1, 18:2 can be detected. The present data also support the findings showing that the fatty acids 16:0, 18:0 and 18:2 dominate the profile of this fungi. Moreover, consistently with our results on *S. californicus* it has been shown that 17 fatty acids, *a*-15:0 and *i*-16:0 are the most abundant FAMES for this bacterium (Suutari *et al.* 2002). Interestingly, the current experimental setting revealed that total microbial biomass was greater

when bacteria and fungi were cultured together compared to separately cultured combination. There were differences between *S. chartarum* fatty acid profile when cultured together or separately indicating differences in physiological state of the microbe.

CONCLUSION AND IMPLICATIONS

These results indicate that co-cultivation affects the biomass and physiological state of the microbes. These microbial interactions may affect the bioactivity of these microbes leading to currently unknown responses in mammalian cells. Thus, the microbial interactions need to be further studied and carefully considered when evaluating the possible health effects associated with exposure in moldy houses.

ACKNOWLEDGMENTS

This study was financially supported by The Academy of Finland and The Juho Vainio Foundation.

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METHOD TO CLASSIFY ENVIRONMENTAL SAMPLES BASED ON MOLD ANALYSES BY QPCR

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ABSTRACT

A total of 82 quantitative PCR (QPCR) assays were used to identify and quantify different indoor molds in dust samples from the homes of six infants suffering from pulmonary hemorrhage and 26 reference homes in Cleveland, Ohio. No significant difference was seen in the total cell quantities (presumed to be primarily spores and conidia) of these molds in the reference home (RH) and pulmonary hemorrhage home (PHH) samples. The assays were categorized based on the ratios of geometric means of measured organisms in the PHH and RH samples. The categories were < 0.5, 0.5 to 2 and > 2 for PHH/RH. Twenty-two assays were identified in the >2 category. The combined quantities of cells detected by these assays comprised only 8% of all detected organisms in the PHH samples but were significantly higher in the PHH samples than in the RH samples ($p=0.001$).

INDEX TERMS: QPCR, pulmonary hemosiderosis, reference homes, dust, Cleveland

INTRODUCTION

A DNA-based technology for quantitative measurement of different species or closely related assay groups of indoor molds has been developed (Haugland *et al.* 1999; Haugland *et al.* 2004; Meklin *et al.* 2004). This technology, called quantitative PCR (QPCR) is now being applied to analysis of various types of environmental samples, including building dust (Roe *et al.* 2000).

In previous work (Meklin *et al.* 2004) we have shown that QPCR and culture-based results are not comparable. Culture methods gave only limited information about the mold species present in building dust and the quantities of colony forming

organisms determined by culture analysis were found to underestimate the total populations of cells present. In this study, QPCR was used to quantify different species or assay groups of molds in dust samples from RH and PHH in Cleveland, OH with the objective of determining whether significant differences occurred in these two sets of homes.

METHODS

Dust samples were collected from six homes of infants with PH and from 26 randomly selected RH showing no evidence of any moisture or mold. All homes were from similar housing stock in the same metropolitan area and sampling of PH and RH was conducted over a similar time frame of approximately 2 years. A square meter area was vacuumed with a cassette filter apparatus (37mm with 0.8 µm filter) connected to an AirCon-2 High Flow Sampler pump (Gilian Instrument Co., Clearwater, FL), calibrated at a flow rate of 10 liter per min. In the PHH, one sample was taken in the infant's bedroom and, in some cases a second sample was also taken in the basement. Each sample was sieved and mixed.

Five mg of each dust sample was extracted by a rapid bead-milling method and QPCR reactions were prepared and performed as previously described (Haugland *et al.* 2002; Haugland *et al.* 2004). Amplification efficiencies and standard curves for each QPCR assay were determined from analyses of serially diluted DNA extracts of conidia and/or spore preparations from pure cultures of verified target species strains as previously reported (Haugland *et al.* 1999; Haugland *et al.* 2004; Meklin *et al.* 2004). Calculation methods for enumerating target organisms in the samples have been extensively described (Brinkman *et al.* 2003; Haugland *et al.* 2002; Haugland *et al.* 2004). The QPCR primer and probe sequences used in this study are available at the website: <http://www.epa.gov/nerlcwww/moldtech.htm>

The different assays were categorized based on whether the ratios of the geometric means (GM's) of measured cell numbers in the PHH/RH samples were < 0.5, 0.5 to 2 or > 2. Statistical analyses of both total and categorized populations were carried out using the 2-tailed Wilcoxon-Mann-Whitney U-test.

RESULTS

The sums of the GM's of all assay target organisms in the PHH and RH were 11,900 and 12,500 cells / 5 mg dust, respectively, and these numbers were not significantly different. *Aureobasidium pullulans*, *Aspergillus penicillioides*, *Cladosporium cladosporioides*, *Epicoccum nigrum* and *Eurotium spp.* dominated the targeted fungal flora of both sets of samples, together comprising approximately 90% of the measured cells in both cases. Each of these species or assay groups, as well as 48 others, occurred in the 0.5 - 2 category of PHH/RH ratios (Table 1). The GM's of 22

species or assay groups were found to be > 2x higher in the PHH dust samples than in those from the RH. The organisms in this category comprised approximately 8% of the detected cells in the PHH samples and were present in significantly higher numbers in these samples than in the RH samples (P=0.001). Conversely, the GM's of only 6 species were > 2x higher in the RH dust samples and the total detected cell numbers of organisms in this category were not significantly different in the two sets of samples.

Table 1. Geometric Mean (GM) of numbers of mold cells in dust from pulmonary hemorrhage homes (PHH) and reference homes (RH) and the GM ratios (PHH/RH).

Species or assay group	PHH GM ^a	RH GM ^a	PHH/RH
<i>Aspergillus caespitosus</i>	0.10	0.13	0.77
<i>Aspergillus carbonarius</i>	0.10	0.10	1.00
<i>Aspergillus candidus</i>	0.53	0.61	0.87
<i>Aspergillus cervinus</i>	0.10	0.20	0.50
<i>Aspergillus clavatus</i> grp. ^b	0.10	0.10	1.00
<i>Aspergillus flavipes</i>	0.10	0.10	1.00
<i>Aspergillus flavus</i> grp. ^c	3.76	2.09	1.80
<i>Aspergillus fumigatus</i> grp. ^d	26.2	2.13	12.3
<i>Aspergillus niger</i> grp. ^e	81.8	69.4	1.18
<i>Aspergillus niveus</i>	0.10	0.10	1.00
<i>Aspergillus ochraceus</i> grp. ^f	164	7.10	23.1
<i>Aspergillus paradoxus</i>	0.10	0.10	1.00
<i>Aspergillus parasiticus</i>	0.10	0.10	1.00
<i>Aspergillus penicillioides</i>	485	294	1.65
<i>Aspergillus puniceus</i>	0.44	0.25	1.76
<i>Aspergillus restrictus</i> grp. ^g	1.86	0.62	3.00
<i>Aspergillus sclerotiorum</i>	1.52	0.74	2.05
<i>Aspergillus sydowii</i>	2.23	1.67	1.33
<i>Aspergillus tamari</i>	0.19	0.29	0.65
<i>Aspergillus terreus</i>	0.10	0.56	0.18

Table 1. Continued

Species or assay group	PHH GM ^a	RH GM ^a	PHH/RH
<i>Aspergillus unguis</i>	36.3	8.31	4.37
<i>Aspergillus ustus</i>	3.50	5.37	0.65
<i>Aspergillus versicolor</i>	75.5	6.42	11.8
<i>Aspergillus wentii</i>	0.10	0.10	1.00
<i>Emericella</i> grp. ^b	0.53	0.18	2.91
<i>Emericella varicolor</i>	0.10	0.10	1.00
<i>Eurotium</i> grp. ⁱ	787	755	1.04
<i>Penicillium atramentosum</i>	0.10	0.15	0.67
<i>Penicillium brevicompactum</i>	30.8	8.48	3.63
<i>Penicillium canescens</i>	1.13	0.29	3.90
<i>Penicillium chrysogenum</i> type 2 ⁱ	48.0	57.7	0.83
<i>Penicillium citreonigrum</i>	0.10	0.10	1.00
<i>Penicillium citrinum</i> grp. ^k	0.19	0.17	1.12
<i>Penicillium digitatum</i>	0.14	0.15	0.93
<i>Penicillium</i> grp1 ^l	0.45	0.41	1.09
<i>Penicillium</i> grp2 ^m	87.3	1.56	56.0
<i>Penicillium coprophilum</i>	0.10	0.10	1.00
<i>Penicillium corylophilum</i>	11.6	3.65	3.17
<i>Penicillium decumbens</i>	0.10	0.10	1.00
<i>Penicillium expansum</i>	0.10	0.10	1.00
<i>Penicillium fellutanum</i> grp. ⁿ	0.20	0.10	2.00
<i>Penicillium glandicola</i>	0.13	0.16	0.81
<i>Penicillium griseofulvum</i>	0.14	0.14	1.00
<i>Penicillium implicatum</i>	0.27	0.20	1.35
<i>Penicillium islandicum</i>	0.10	0.15	0.67
<i>Penicillium italicum</i>	0.10	0.10	1.00
<i>Penicillium melinii</i>	0.10	0.10	1.00
<i>Penicillium miczynskii</i>	0.10	0.10	1.00

Table 1. Continued

Species or assay group	PHH GM ^a	RH GM ^a	PHH/RH
<i>Penicillium olsonii</i>	0.52	0.23	2.26
<i>Penicillium oxalicum</i>	0.56	1.14	0.49
<i>Penicillium purpurogenum</i>	1.51	0.97	1.56
<i>Penicillium raistrickii</i>	0.10	0.10	1.00
<i>Penicillium restrictum</i>	0.17	0.15	1.13
<i>Penicillium roquefortii</i>	0.32	0.19	1.68
<i>Penicillium sclerotiorum</i>	0.20	48	0.42
<i>Penicillium simplicissimum</i> grp. ^o	0.15	0.10	1.50
<i>Penicillium spinulosum</i> grp. ^p	160	11.1	14.4
<i>Penicillium variable</i>	16.0	4.10	3.90
<i>Paecilomyces variotii</i>	14.3	5.24	2.73
<i>Aureobasidium pullulans</i>	5100	3600	1.42
<i>Acremonium strictum</i>	3.64	6.61	0.55
<i>Alternaria alternata</i>	93.5	290	0.32
<i>Chaetomium globosum</i>	5.69	4.38	1.30
<i>Cladosporium cladosporioides</i> - Type 1	1490	2540	0.59
<i>Cladosporium cladosporioides</i> - Type 2	220	234	0.94
<i>Cladosporium herbarum</i>	91.8	229	0.40
<i>Cladosporium sphaerospermum</i>	127	45.8	2.77
<i>Epicoccum nigrum</i>	2430	4110	0.59
<i>Memmoniella echinata</i>	0.14	0.12	1.17
<i>Mucor and Rhizopus</i> grp. ^a	68.6	107	0.64
<i>Rhizopus stolonifer</i>	0.62	2.79	0.22
<i>Scopulariopsis brevicaulis</i>	4.48	1.49	3.01
<i>Scopulariopsis chartarum</i>	6.22	1.22	5.10
<i>Stachybotrys chartarum</i>	5.64	1.05	5.37
<i>Trichoderma asperellum</i> ^t	0.26	0.14	1.85

Table 1. Continued

Species or assay group	PHH GM ^a	RH GM ^a	PHH/RH
<i>Trichoderma harzianum</i>	0.57	0.16	3.56
<i>Trichoderma longibrachiatum</i> grp. ^s	0.38	0.38	1.00
<i>Trichoderma viride</i> grp. ^t	31.9	0.52	61.3
<i>Ulocladium atrum</i>	0.20	0.12	1.67
<i>Ulocladium chartarum</i>	0.10	0.10	1.00
<i>Ulocladium botrytis</i>	0.80	0.66	1.21
<i>Wallemia sebi</i>	166	46.9	3.54

- a Geometric mean of calculated cell numbers per 5 mg dust. Values of 0.10 = none detected
- b Includes *A. clavatus* and *A. giganteus*
- c Includes *A. flavus* and *A. oryzae*
- d Includes *A. fumigatus* and *Neosartorya fischeri*
- e Includes *A. niger*, *A. awamori*, *A. foetidus* and *A. phoenicis*
- f Includes *A. ochraceus* and *A. ostianus*
- g Includes *A. restrictus*, *A. caesillus* and *A. conicus*
- h Includes *E. nidulans*, *E. quadrilineata* and *E. rugulosa*
- i Includes *E. amstelodami*, *E. chevalieri*, *E. herbariorum*, *E. rubrum* and *E. repens*
- j Dominant subgroup of species
- k Includes *P. citrinum*, *P. sartoryi* and *P. westlingi*
- l Includes *P. aurantiogriseum*, *P. freii*, *P. hirsutum*, *P. polonicum*, *P. tricolor*, *P. verrucosum* and *P. viridicatum*
- m Includes *P. crustosum*, *P. camembertii*, *P. commune*, *P. ebinulatum* and *P. solitum*
- n Includes *P. fellutanum* and *P. charlesii*
- o Includes *P. simplicissimum* and *P. ochrocloron*
- p Includes *P. spinulosum*, *P. glabrum*, *P. lividum*, *P. pupurescens* and *P. thomii*
- q Includes *M. amphibiorum*, *M. circinelloides*, *M. hiemalis*, *M. indicus*, *M. mucedo*, *M. racemosus*, *M. ramosissimus*, *R. azygosporus*, *R. homothallicus*, *R. microsporus*, *R. oligosporus* and *R. oryzae*
- r Includes *T. asperellum* and *T. hamatum*
- s Includes *T. longibrachiatum* and *T. citrinoviride*
- t Includes *T. viride*, *T. atroviride* and *T. koningii*

DISCUSSION

In this study, the total mold cell quantities in PHH and RH dust samples were essentially the same. However, the 22 mold groups in the >2 PHH/RH ratio category allowed us to statistically define a difference between PHH and RH samples. The >2 ratio category includes molds often associated with water damage, like *S. chartarum* and *T. viride* but also some of the slightly or moderately xerophilic species, including *A. fumigatus*, *A. ochraceus*, *A. versicolor* and *Penicillium* species such as *P. crustosum*, *P. commune*, *P. echinulatum* or *P. solitum* (Flannigan, Miller, 2001). Many of these species are known for their pathogenicity and/or toxicity.

The relationship between indoor molds and PH remains uncertain. This study illustrates one means by which QPCR analyses may be useful in further evaluating the association between indoor molds and this illness. The rapid identification of various species that are significantly elevated in PHH dust samples compared with reference homes should facilitate more timely and rationally focused monitoring for exposures to these different candidate etiological agents.

More generally, our results suggest that QPCR analysis for selected species in dust samples may provide a means for rapidly identifying buildings with cryptic water damage and mold incursions. Species occurring in the highest abundance may not be the best indicators of such buildings.

CONCLUSION AND IMPLICATIONS

QPCR analyses for specific molds in house dust identified a discreet group of mold species that distinguished the homes of PH victims from reference homes based on their occurrence in significantly higher numbers in the former. While further studies are needed to establish the general applicability of this home classification procedure, the underlying QPCR technology has been demonstrated to be a useful tool for studying the relationship between indoor molds and building related illnesses and in identifying buildings with abnormal mold populations.

ACKNOWLEDGEMENTS

This research was supported by the United States Environmental Protection Agency "Children at Risk" Program.

The United States Environmental Protection Agency through its Office of Research and Development partially funded and collaborated in the research described here under Cooperative Agreement CR827942-01-0 with Case Western Reserve University. It has been subjected to Agency review and approved for publication.

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QUALITATIVE IDENTIFICATION OF *MERULIPORIA INCRASSATA* USING REAL TIME POLYMERASE CHAIN REACTION (PCR)

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ABSTRACT

The increased concern over building wood structural damage caused by wood decay fungi has hastened the need for reliable and accurate identification of the cause, particularly *Meruliporia incrassata* (*Poria incrassata*), a water-conducting brown-rot fungus. Without the presence of fruiting bodies (basidiomata), positive identification of wood decay fungi based on the morphological characteristics of the vegetative structures is impractical and unreliable. In order to provide a rapid and accurate identification, the sequences of rRNA gene were used to develop genetic amplification and identification using real time PCR. Three sets of primer pairs and probes were selected and evaluated for specificity and sensitivity of detection. These primer pairs amplify only the DNA extracted from a pure culture of *M. incrassata* but not *Serpula lacrymans*, *Serpula himantioides*, *Sistotrema brinkmannii* and other wood decay fungi. In addition, samples collected from different residential buildings tested positive only if they were infested and decayed by *M. incrassata* but not by *S. lacrymans*. The results demonstrate that real time PCR analysis is an useful method for providing an accurate confirmation of *M. incrassata*-infested wood and a fast detection system for monitoring, preventing and controlling wood decay.

INDEX TERMS: PCR, Wood Decay, Brown Rot, rRNA gene

INTRODUCTION

Meruliporia incrassata (*Poria incrassata*) is a wood decay fungus occurring on wood, mainly conifers, and especially on structural timbers of buildings (Lowe, 1966). Several broadleaf woods are hosts also. Hosts include, *Pinus*, *Pseudotsuga*, *Quercus*, *Sequoia*, *Sequoiadendron*, *Taxodium*, *Tsuga*, *Robinia*, and *Magnolia* (Farr *et al.* 1989).

Meruliporia incrassata causes massive damage on floors and walls away from obvious sources of moisture. *Meruliporia incrassata* often occurs in new or remodeled houses and can cause extensive damage within two to three years.

Common water or moisture sources in buildings, which may lead to the development of this fungus, include water leaks and wood in close contact with soil infested by the fungus. One of the reasons for making this fungus very destructive is its ability to transport water for 50 to more than 100 cm through mycelia fans, and mycelial strands or rhizomorphs, allowing it to transport water from the soil or other water sources to the wood (Verall 1968).

Meruliporia incrassata may produce a dry rot form of attack as do *Serpula lacrymans* and several wood decay fungi. However without the presence of basidiomata, identification of this fungus, based on morphological characteristics, is difficult and impractical. In indoor environments the basidiomata rarely develop on building materials. It is therefore necessary to develop a reliable and accurate method to identify *M. incrassata* without the presence of its basidiomata for its prevention and remediation.

The objective of this study is to develop species-specific primer pairs and probes for rapid and accurate identification of *M. incrassata* using real time PCR.

METHODS

Cultures and DNA Extraction and Purification: The culture collections of brown rot fungi, *Meruliporia incrassata* (Mad-563), *Serpula lacrymans* (ATCC-36335) and *Serpula himantioides* (RLG-12941) were kindly provided by the forest products laboratory, United States Department of Agriculture (Madison, WI 53705). Malt extract agar (2% MEA, pH4.7, Difco laboratories, Detroit, MI, U.S.A.) was used to maintain and subculture all the isolates of brown-rot fungi and other fungi. All fungal cultures were incubated at 23°C for 5-10 days.

Mycelial samples from 5 to 10-day-old colonies were harvested with a surgical scalpel from the surface of pure cultures. Genomic DNAs were released from mycelia using a glass bead-beating method (Haugland *et al.* 1999). Briefly, mycelia were combined in an extraction tube with an internal reference (*Geotrichum candidum*, UAMH 7863), acid-washed glass beads (G-1277; Sigma, St Louis, MO, U.S.A.), extraction buffer (lysis buffer - 100 µL and binding buffer - 300 µL; an Elu-Quik DNA purification kit from Schleicher and Schuell, Keene, NH, U.S.A.) and were shaken using a beadbeater (Biospec Products, Bartlesville, OK, U.S.A.) at maximum rate for 1 min. DNAs were recovered and further purified with the DNAeasy tissue kit (Qiagen, Inc., Valencia, CA, U.S.A.). The concentration and purification protocols were performed following manufacturer's specifications.

Sample Process: Two methods were used for removing fungal cells from the wood/particulate samples. The first was a direct extraction from field samples. Briefly, the suspicious fungal structures picked off the wood or small pieces of decayed wood from field samples were added to extraction tubes for DNA extraction and purification as described. Another method was sample pooling. Briefly, a portion of each wood sample or various pieces of wood were selected and added to a 15 mL centrifuge tube containing 10 mL of 0.05% Tween solution. Tubes were vigorously vortexed to release fungal cells from the wood. The supernatant was collected and washed and the pellet was transferred to an extraction tube for DNA extraction and purification as described.

A known quantity of *Geotrichum candidum* was added to the extraction buffer to serve as an internal reference (IR). By adding an internal reference to the extraction buffer, the overall success of the DNA extraction, DNA recovery and PCR amplification can be monitored.

Primer Design and PCR amplification: *Meruliporia incrassata* sequences for rRNA gene, particularly the internal transcribed spacers (ITS) between 18S rRNA gene and 28S rRNA gene, 18S-ITS1-5.8S-ITS2-28S were obtained from GenBank and compared against all other sequences available on-line with the Basic Local Alignment Search Tool algorithm (BLAST, national Center for Biotechnology Information, National institutes of Health). Primers and probes were designed using Primer Express Software (Applied Biosystems) and were synthesized by Integrated DNA Technologies, Inc. Coralville, IA, U.S.A..

The ABI Prism 7000 Sequence Detection System and 5700 SDS (7000 SDS, 5700SDS; Applied Biosystems) were used for PCR analysis. With the use of Applied Biosystems reagents, the amplification conditions were as follows: 1X TaqMan master mix (with AmpErase Uracyl N-Glycosylase); 0.02 mg/mL BSA; 1 μ M of each primer; 0.8 μ M Probe and 5 μ L fungal (or tested) DNA template for a total reaction volume of 25 μ L. The thermal cycling conditions, as default program setting, consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 second at 95°C and 1 min at 60°C. *Meruliporia incrassata* (Mad-563) DNA was used for testing the primers and probes of *M. incrassata*. *Serpula lacrymans* (ATCC-36335) DNA, *S. himantioides* (RLG-12941) and other fungal species were used to test the specificity (non-specific cross-amplification) of the primer and probes designed.

RESULTS

Selection of sequence-specific PCR primers: Primer and Probe selections were performed by computing programs and by manual inspection of the aligned sequences. The sequence of *Meruliporia incrassata* for the ITS1-5.8S-IT2 was

obtained from GenBank (GenBank accession # AJ419913) and compared against all other sequences on-line with BLAST. BLAST results revealed a high degree of sequence identity to other wood decay fungi in 5.8S but not in the sequence of ITS1 and ITS2 regions. To select the sequence-specific primers and probes for targeting *M. incrassata* using real time PCR (TaqMan chemistry), the available rRNA gene sequences of brown rot fungi were selected. The sequences of rRNA genes of the brown rot fungi, *Meruliporia incrassata* (Accession # AJ419912, isolate P261: Accession # AJ419913), *Serpula lacrymans* (isolate U.S.A.94: Accession # AF335276, isolate s27: Accession # SLA419910) and *S. himantioides* (Accession # SHI419911) were aligned to determine the sequence variations in ITS1 and ITS2. Four high variable regions were identified for the selection of primers and probes using Primer Express Software (Applied Biosystems). One set (MI-5) of the primers and probe was selected from the ITS1 regions with the amplicon of 99-bp (Fig. 1); the forward primer MI-86-105F (5'-GTGCTGGCCACCTTTATCTTG -3'), reverse primer MI-165-184R (5'-ATACGTCGGCGTGTGATGAA -3') and the probe MI-119-143P (5'-FAM-ACACACACCAGTGCACCTGCCGTAG -TAMRA-3') in between. Two sets of primers and probes were obtained from ITS2 variable regions, MI-3 and MI-6, which produced amplicons of 71-bp and 98-bp respectively. The primers/probes for the MI-3 set are MI-496-514F: 5'-GTGTTGCTCGGCGTGATA-3', MI-520-544P: 5'-6FAM-CGTCGTGTCTGGCGTGCAAGTAGGA-TAMRA-3', MI-548-566R: 5'-GACGGTTGTGGGCAAAAGG-3'. Those for the MI-6 set are MI-366-391F: 5'-TGCCTGTTTGAGTGTTCAT-TAAATTCT-3', MI-398-423P: 5'-6FAM-CTCCAATTCGTTTTTGACGTGGGCTT-TAMRA-3', MI-445-463R: 5'-AGCCGACCACACAAGGTCA-3' (Fig. 1)

Figure 1. Illustration of a typical fungal rRNA gene and the positions of the designated sets of primers/probes for the specific detection of *Meruliporia incrassata* using real time PCR (TaqMan assay). The sequence number shown on the graph was derived from *Meruliporia incrassata* (Accession # AJ419912, isolate P261).

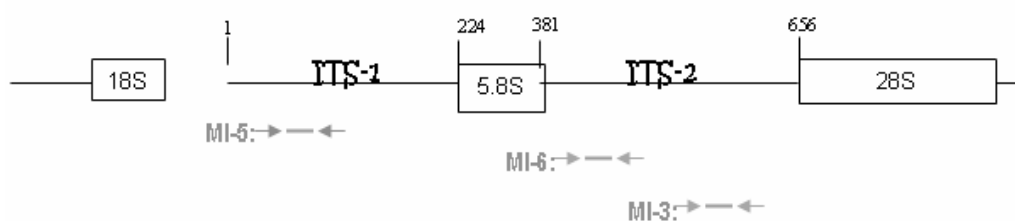


Table 1. Specificity testing of Wood decay fungi using designated primer pairs and probes of *Meruliporia incrassata*.

Organism	PCR Results		
	MI-3	MI-5	MI-6
<i>Meruliporia incrassata</i> (Mad-563)	+	+	+
<i>Serpula lacrymans</i> (ATCC-36335)	–	–	–
<i>Serpula himantioides</i> (RLG-12941)	–	–	–
<i>Sistotrema brinkmannii</i> (STL-P&K isolate 250)	–	–	–

PCR performance and Primers/probes specificity: To evaluate the specificity and sensitivity of the primers and probes, MI-3, MI-5 and MI-6 were set up to amplify DNA extracted from the pure culture of *M. incrassata* and other wood decay fungi (table 1). In conjunction with the fluorogenic nuclease assay and sequence detector, all three sets were able to amplify DNA extracted from the pure culture of *M. incrassata* as evidenced by the detected levels of fluorescence. Under the same conditions, those sets fail to amplify DNA templates from pure cultures of other brown rot fungi, *S. lacrymans*, *S. himantioides* and other wood decay fungi, *Sistotrema brinkmannii* (STL-P&L isolate 250), indicating the target specificity of the designated primer pairs and probes. Sensitivity of detection was tested with dilutions of *M. incrassata* DNA in order to select one set for further testing. We found that MI-3 was slightly more sensitive than MI-5 and MI-6 (data not shown). MI-3 was, therefore, selected for later tests. To determine whether cross amplification occurs, other fungal species were also examined. There was no PCR product observed using DNA extracted from those fungal species, which are most commonly found in water-damaged related indoor environments (table 2).

Table 2. Cross-amplification test on common indoor fungi using MI-3 set.

Organism	MI-3	Organism	MI-3
<i>Acremonium strictum</i> (ATCC 34717)	–	<i>Eurotium amstelodami</i> (NRRL 90)	–
<i>Alternaria alternate</i> (STL-PK isolate)	–	<i>Chaetomium globosum</i> (ATCC 32404)	–
<i>Aspergillus flavus</i> (NRRL 16883)	–	<i>Cladosporium cladosporioides</i> (ATCC 16022)	–
<i>Aspergillus fumigatus</i> (NRRL 163)	–	<i>Memnoniella ecbinata</i> (UAMH 6594)	–
<i>Aspergillus niger</i> (ATCC 16888)	–	<i>Paecilomyces varioti</i> (ATCC 22319)	–
<i>Aspergillus sydowii</i> (STL-PK isolate)	–	<i>Penicillium brevicompactum</i> (STL-PK isolate)	–
<i>Aspergillus ustus</i> (STL-PK isolate)	–	<i>Penicillium chrysogenum</i> (STL-PK isolate)	–
<i>Aspergillus versicolor</i> (STL-PK isolate)	–	<i>Stachybotrys chartarum</i> (UAMH 6417)	–
<i>Aspergillus ochraceus</i> (NRRL 398)	–	<i>Trichoderma viride</i> (UAMH 6280)	–

ATCC, American Type Culture Collection; UAMH, University of Alberta Microfungus Collection and Herbarium; NRRL, Northern Regional Research Laboratory, STL-PK, STL-P&K Microbiology Service Inc.

To apply the PCR analysis to real field samples from environmental sources, we collected and tested the samples from residential buildings known to be infested/and decayed by *M. incrassata*, *S. lacrymans* or other wood decay fungi. The results showed that the positive fluorescence detection was only observed on the PCR reactions in which the DNA templates were extracted from the *M. incrassata*-infested samples but not from *S. lacrymans*-infested samples or decayed wood infested by other fungi (table 3). The results demonstrate that the primer pairs and probes used in the study can accurately and specifically identify the presence of *M. incrassata* from *M. incrassata*-infested wood.

Table 3. PCR results obtained from field samples

Samples	Identification	PCR Results (MI-3)	Locations
Bulk	<i>Meruliporia incrassata</i>	+	Downey, CA
Bulk	<i>Serpula lacrymans</i>	–	Highland, NC
Bulk	<i>Megacollybia platyphylla</i>	–	Hershey, PA
Bulk	<i>Poria sp.</i>	–	Chesapeake, VA

DISCUSSION

A 1% weight loss of wood resulting from decay can lead to 50% loss in strength measured as toughness (Richards 1954). Proper diagnosis of early decay and the causative agents allow for appropriate remedial treatments to arrest decay prior to loss of structural integrity. Many techniques have been successfully developed for detecting wood decay and its causative agents. Those techniques include visual and microscopic examination (Wilcox 1964), culturing of fungi from wood (Nobles 1965), direct chemical staining (Eslyn 1979) and immunological detection (Goodell *et al.* 1986, 1988). The culture method is relatively simple, but is tedious and time-consuming. Advances in immunological detection methods held promise for detecting incipient wood decay, albeit the cross-reactivity of the reaction was noted (Clausen 1997). PCR is a nucleic acid technique based on amplification of a selected DNA sequence to levels that can be detected instrumentally. It is known to be very accurate, sensitive and reliable and is now a common practice for genetic identification, in addition to the traditional culture method.

Real time PCR (with the use of a fluorogenic probe), on the other hand, not only increases the specificity and sensitivity of the detection but also provides a quantitative measurement for the detection. In this study, the designated primer pairs and probes have proven to be very accurate and specific in detecting the presence of *M. incrassata* in wood, indicating that real time PCR analysis is a very useful method to accurately confirm *M. incrassata*-infested wood.

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A NEW ECOLOGICAL NICHE FOR THE PATHOGENIC YEAST, *CRYPTOCOCCUS NEOFORMANS* VAR. *GATTII*, IN A TEMPERATE CLIMATE ZONE

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INTRODUCTION

Cryptococcosis is an infection caused by an encapsulated, basidiomycetous yeast, *Cryptococcus neoformans*. The route of entry for infection for this organism is through the lungs, with possible systemic spread via the circulatory system to the brain and meninges. There are four cryptococcal serogroups associated with disease in humans and animals, distinguished by capsular polysaccharide antigens. *C. neoformans* is grouped into three varieties: *grubii* (serogroup A), *neoformans* (serogroup D) and *gattii* (serogroups B and C). (Franzot 1999; Kwon-Chung 1992).

Cryptococci of serogroups A and D have a world-wide distribution and are particularly associated with soil and weathered bird droppings, although the birds themselves are not affected by the organism (Kwon-Chung 1990, 1992; Hubalek 1975). *C. neoformans* serogroups A and D have become a leading cause of life threatening infection in immunocompromised persons (Kwon-Chung 1992).

In contrast, *C. var. gattii* (serogroup B) may infect a variety of mammalian hosts with intact immune systems. *C. var. gattii* has been reported to have a limited habitat in the tropics or sub tropics, and to colonize specific tree hosts, the river gum eucalypts (*Eucalyptus camaldulensis* and *E. tereticornis*) (Ellis 1990a). However, beginning in 1999, increasing numbers of cases of human and animal disease associated with *C. var. gattii* were referred to the Central Laboratory for Veterinarians and the British Columbia Centre for Disease Control. Cases of cryptococcosis caused

by *C. var. gattii* were confirmed in hosts with no other travel history than to have lived or to have visited the eastern coast of Vancouver Island, Canada.

From January 1999 – June 2003, the number of confirmed human cases was 77 with four deaths directly attributable to *C. var. gattii* infection. Globally, the annual incidence rate of cryptococcosis is 1.5 per million population. The cases in British Columbia (BC) represent an average incidence rate of 36 cases per million, which is over four times the rate of 8.5 cases per million population in tropical northern Australia where cryptococcosis associated with *C. var. gattii* is endemic (Sorrell, 2001).

Since January 2003 there has been a sudden increase in the number of cases of cryptococcosis reported in companion animals, with some 40 new cases this year in cats, dogs, ferrets and one bird. A number of these human and animal cases, particularly in cats, confirmed as *C. var. gattii* cryptococcosis, have no travel history to tropical or sub-tropical areas, no exposure to Eucalypts, and very limited exposure to the outdoor environment.

Objective: A search was initiated in BC for sources of exposure to *C. neoformans* var. *gattii*, with particular emphasis on airborne exposures to account for the human and animal cases of cryptococcosis reporting limited exposure to any outdoor or wooded environments.

METHODS

Sample sites: Samples were taken from the homes and environs of cases (human and animal). All trees or homes were mapped using a GPS unit (Etrex). Sampling locations that were positive for the recovery of *C. neoformans* were tagged and repeatedly sampled throughout a calendar year. The abodes of animal and human cases of cryptococcosis were mapped using GIS coordinates, with the majority of cases resident on the eastern coast of Vancouver Island

Swab samples

Sterile, cotton tipped swabs with accompanying Amies transport media (Starswab II™) were used to recover cryptococci from trees. Swabs were dipped into the media to wet the swab which was then rubbed into limb holes, natural cracks in bark, or holes in bark made by birds or animals. The swab was then inserted into the transport media and placed in a cooler for transport to the laboratory. In homes, the swabs were also used to collect material from house plants, window ledges, or areas where afflicted pets spent time. In the laboratory, the swabs were streaked onto a differential agar medium (Niger seed agar). Ground niger seed in the agar provided caffeic acid as a substrate from which phenoloxidase-positive

organisms form melanin, allowing cryptococci belonging to serogroups A, D, B or C to be visualized. Plates were incubated at 30°C for 48 hours to 7 days. Cryptococci which were brown on Niger seed agar were purified and transferred to Canavanine-Glycine-Bromothymol Blue (CGB) agar (Kwon-Chung 1982). Growth of cryptococci belonging to serogroups B and C turn the medium blue in contrast to serogroups A and D which do not. Cultures conforming to cultural and microscopic characteristics were serotyped using purified antibodies to capsular antigens (Crypto Check, Iatron Laboratories).

Air samples: (1) An Andersen six-stage sampling head was fitted with 100 mm plates containing 45 mL of niger seed agar. Air was drawn through the sampling head at 28.3 lpm for 15 min using a portable AirCon II pump. (2) A Reuter Centrifugal Sampler (RCS) was fitted with a 34 compartment strip filled with niger seed agar. RCS samples were taken for 4 min at an effective flow rate of 40 lpm. Agar media were returned to the lab and incubated as described.

Soil samples: Zip lock bags were used to collect surface soil samples. In the laboratory, approximately 2 grams of soil was distributed into sterile 50 mL centrifuge tubes and 10 mL sterile water added. The tubes were vortexed, then the contents allowed to settle for 10 min. Duplicate plates of niger seed agar were inoculated with 0.1 mL each and processed as described above.

PCR: Molecular fingerprinting was performed on human, animal, and environmental isolates using polymerase chain reaction (PCR)-URA5 restriction fragment length polymorphism (RFLP) (Meyer 1999).

RESULTS

Cryptococcus neoformans was isolated from swab, air and soil samples. Table 1. summarizes the number of initial, investigative swab samples that were positive for *C. neoformans* var. *gattii* culture.

Cryptococcus neoformans var. *gattii* was isolated from air in the environs of culture positive trees. Although there were seasonal differences in concentration, the organism was airborne in all seasons (Table 2).

Table 1. results of initial swab samples of 1528 trees.

Tree species	Number examined	Number positive	% positive
Alder (<i>Alnus</i>)	174	22	13
Arbutus (<i>Arbutus</i>)	82	9	10
Ash (<i>Fraxinus</i>)	2	1	50
Birch (<i>Betula</i>)	5	0	0
Cherry (<i>Prunus</i>)	19	2	11
Cedar (<i>Thuja</i>)	166	17	10
Dead stump	8	1	13
Douglas fir (<i>Pseudotsuga</i>)	499	69	14
Eucalyptus (<i>Eucalyptus</i>)	23	0	0
Fir (<i>Abies</i>)	65	10	15
Fruit trees (various)	13	0	0
Garry Oak (<i>Quercus</i>)	65	14	22
Hemlock (<i>Tsuga</i>)	27	0	0
Maple (<i>Acer</i>)	122	9	7
Pine (<i>Pinus</i>)	38	2	5
Poplar (<i>Populus</i>)	16	0	0
Spruce (<i>Picea</i>)	23	1	4
Willow (<i>Salix</i>)	10	0	0
Other (or unknown)	171	4	2

Table 2. Airborne concentration of *C. neoformans* (Serogroup B) near culture positive trees in BC.

Season	n	GM ^a	GSD ^b	Range
		CFU/m ³		CFU/m ³
Summer (June – August)	27	30.5 ^c	11.6	0 – 2692
Fall (September – November)	23	6.7 ^d	5.5	0 – 436
Winter (December - February)	25	1.2 ^e	1.8	0 – 7
Spring (March – May)	21	5.3	6.2	0 – 550

a geometric mean

b geometric standard deviation

c Summer GM significantly different from Fall, Winter and Spring GMs ($p < 0.001$) by Scheffe post hoc test.

d Fall GM significantly different from Winter GM ($p < 0.001$)

e Winter GM significantly different from Spring GM ($p < 0.001$).

C. neoformans was also isolated from soil under the canopies of trees or where trees had been removed for development.

Table 3. *C. neoformans* serogroup B concentration in soil by geographic location.

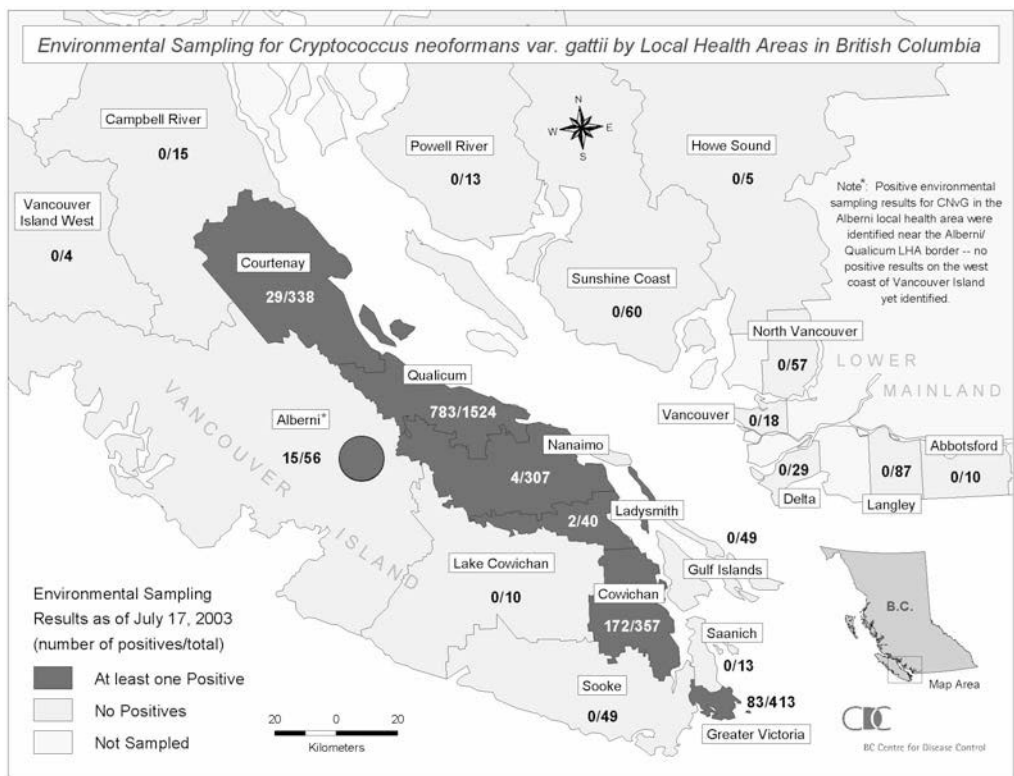
Location	n	GM	GSD	range
		CFU/gram soil	CFU/gram soil	CFU/gram soil
(1) Victoria (southern tip of Vancouver Island)	45	16.6	18.2	0 – 3236
(2) Cowichan (50 km north of Victoria)	71	245	69.0	0 – 331131
(3) Nanaimo (100 km north of Victoria)	56	0	0	0
(4) Parksville (125 km north of Victoria)	271	43.7	1.6	0 – 338844
(5) Courtenay (200 km north of Victoria)	58	3.3	12.0	0 – 4365
(6) Port Alberni (40 km west of Parksville)	15	4.1	6.3	0 – 71
(7) Gulf Islands (11 – 15 km west of Cowichan)	43	0	0	0
(8) Mainland BC (50 km east of Vancouver Island)	41	0	0	0

Epidemiology: Demographics: Mean case age at diagnosis was 59.7 years (range 20 – 82; SD 13.5). Fifty-eight percent of cases were male. Sixty percent were retired or unemployed at the time of their illness. Thirty matched case-control sets were interviewed. Risk factors for a diagnosis of cryptococcosis included: prior diagnoses of pneumonia (OR 2.71, 95%CI 1.05 – 6.98) or other lung problems (OR 3.21; 95% CI 1.08 – 9.52), or use of systemic corticosteroids (OR 8.11; 95% CI 1.74 – 37.8).

Animal cases had a wide distribution of ages from 9 months – 13 years. Risk factors have not been identified for animal cases.

PCR: Clinical cultures from immunocompetent humans and animals, and environmental isolates all belonged to Serogroup B. PCR-RFLP analysis of clinical and environmental isolates revealed two genetic variants, VGII (93%) and VGI (7%).

Figure 1. GIS mapping of tree location sampled for *C. neoformans* var. *gattii*.



CONCLUSION

This is the first description of recovery of the pathogenic basidiomycetous yeast, *Cryptococcus neoformans* from the environment in a temperate climate zone.

Although the prevalence of cryptococcosis is low (36 per million), the disease is debilitating and requires many months of antifungal therapy to resolve. The organism is not infectious from human to human or from animal to human contacts. The ecological niche of basidiomycetous fungi in decaying wood has led researchers to theorize that *C. var. gattii* requires a tree host to become pathogenic to mammals, probably through the formation of *basidiospores* (Ellis, 1990b).

Cases of animal or human cryptococcosis caused in BC by *C. neoformans* var. *gattii* have been recognized since 1999. However, searches through stored cultures have not found any Serogroup B organisms prior to this outbreak, suggesting this is an

emerging infectious disease for this area. To date, no environmental isolates have been found in British Columbia other than on the east coast of Vancouver Island. The east coast of the island is characterized by soil and vegetation of the Coastal Douglas Fir biogeoclimatic zone. The east coast of Vancouver Island has lower average rainfall and milder winters than the remainder of the east coast of the island or the west coast of the Mainland, which is characterized primarily by soil and vegetation of the Mountain Hemlock zone.

In tropical and sub-tropical zones *C. neoformans* var. *gattii* colonizes unique tree hosts (e.g. Eucalypts in Australia, India, Brazil, and Columbia). In contrast, *C. neoformans* var. *gattii* in BC has colonized a wide variety of trees, both deciduous and coniferous. The organism has consistently been recovered from swab cultures taken over a period of a year. In the tropics the organism is airborne only when the colonized trees are blooming (or pollinating). Although the concentration in air is higher in the summer than other seasons on the east coast of Vancouver Island, we have recovered airborne organisms in all months of the year.

The primary ecological niche of this organism is most likely the mixture of soil and tree debris. High concentrations of the organism have been consistently recovered from top soil. The high concentration of cryptococci indicates extremely successful competition with the rich diversity of microflora that colonize the rhizome spheres of vegetation. This suggests that it is less likely that the organism is a transient visitor to the island. Studies are underway to characterize the soil chemistry for comparison with soil from the Mountain Hemlock zone.

One of the puzzling aspects of this outbreak has been how home-bound humans or indoor pets have contacted the organism. The east coast of Vancouver Island is richly forested. Because of the mild climate, this area is experiencing a burgeoning population boom with attendant increase in new home construction in areas that were formerly forested. Home air conditioning units are rare in single family dwellings here, and fungal propagules can easily enter open windows. Approximately 6 – 10% of the airborne propagules were $\leq 3.3\mu\text{m}$ (data not shown) which are small enough to enter the deep lung. The organism has been recovered from the soles of shoes of the research staff after sampling trips into culture positive areas suggesting that the organism can be carried indoors by fomites.

In conclusion, a new ecological niche for *C. neoformans* var. *gattii* has been described. Research is continuing to characterize other determinants of exposure to this organism (e.g. climate, soil or wood product movement) in order to reduce the risk of environmentally acquired cryptococcosis for susceptible populations.

Acknowledgements:

This study was funded in part by Michael Smith Foundation for Health Research (K. Bartlett, Scholar), the British Columbia Lung Association, and Workers' Compensation Board of British Columbia.

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INDIVIDUAL EXPOSURE ASSESSMENT IN RESIDENTS NEAR LARGE SCALE COMPOSTING SITES

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ABSTRACT

In the scope of a cross sectional study relevant bioaerosol pollution was measured in residential air near a large scale composting site in 1997. Irritative airway-related complaints were associated with this exposure in next residents. In 2002 the determination of specific IgG antibodies against actinomycetes and filamentous fungi was offered to these residents in the scope of individual exposure assessment. This was also done in respective control groups. A total of 112 subjects participated in the blood sampling. Analysis of variance of the different types and sums of antibody concentrations was not associated with the previous residential exposure, history of hay fever or other allergies. Women showed significant higher total antibody levels against actinomycetes while there was no difference concerning the region of home (rural or urban). Results indicate high variances in specific IgG antibody levels against actinomycetes.

INDEX TERMS: Specific IgG antibodies, actinomycetes, fungi, residential bioaerosols, exposure assessment

INTRODUCTION

The development of new technologies in the waste treatment industries due to modern disposal concepts has led to a growing amount of recyclable waste in the last ten years, e.g., composting of organic household waste. In the course of processing of organic household waste in large scale composting facilities health relevant molds and actinomycetes are generated and accumulated in compost material become airborne as vegetative cells or spores by movement of the material and

lead to bioaerosol contamination of respective work places and emissions from the sites. (Lacey, Crook, 1988; Millner, 1995). This has led to a growing interest in the assessment of possible adverse effects on the health of employees in waste treatment industries and residents living near large scale composting facilities.

Prior studies dealing with the health relevance of microbiological aerosols for employees in waste processing industries showed a higher rate of respiratory and general health symptoms and diseases (Bünger *et al.* 1998; Marth *et al.* 1997). Severe diseases, e.g. hypersensitivity pneumonitis or severe toxic reactions (toxic pneumonitis, organic dust toxic syndrome (ODTS)) were reported in workers and a private person following direct contact with compost (Lacey, Crook, 1988; Vincken, Roels, 1984; Weber *et al.* 1993; Brown *et al.* 1995). The immunological reactions following the inhalative occupational exposure were shown in significantly increased concentrations of antibodies against thermophilic actinomycetes and molds among workers in waste processing plants (Bünger *et al.* 2000).

In the scope of a cross sectional study in 1997 relevant bioaerosol pollution had been measured in residential air near a large scale composting site (Herr *et al.* 2003). The site was closed and follow-up survey of the residents' health was performed in 1999 and 2002. In 2002 the determination of specific IgG antibodies against actinomycetes (*Streptomyces thermovulgaris*, *Saccharomonospora viridis*, *Saccharopolyspora rectivirgula*, *Saccharopolyspora hirsuta*) and molds (*Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus versicolor*, *Aspergillus sydowii*) was also offered to residents in an attempt to assess previous individual exposure.

METHODS

Study population: For the preceding cross sectional study in 1997 a composting facility had been chosen considering topographical, and meteorological as well as technical aspects (site not completely closed off, turnover of approximately 12,500 Mg per year). The selected facility, located in an urban area, had started to process yard trimmings in 1991 and additionally organic household waste in 1995. Criteria for the selection of the specific study groups were similarity of population pattern, residential area (size of households, intensity of road traffic, frequency of gas stations and industrial sites), as well as the lack of other sources of bioaerosol exposure (sewage plants etc.) in the proximity of the residential area. The residential area close to the composting plant was located at a distance from 150 to 500 m downwind. Measurements of viable airborne microorganisms in residential air were performed during the ongoing epidemiological study. The site was closed down after this study in September 1997.

In 2002 a follow up was conducted: about 400 of the (formerly) exposed and 400 subjects without exposure living in different areas of the same governmental dis-

tract were selected. Additionally residents 1500 m from another composting site in a rural area were investigated. Residents of another village in the same district were selected as “not exposed” control group.

Environmental health questionnaire: For the assessment of self-reported health (complaints, symptoms, and lifetime prevalence of physician diagnoses) in 1997 an environmental health questionnaire had been developed relating to items of national and international studies, for example ISAAC (Anonymous, 1998). Prevalence of respiratory, eye related, and general health complaints, as well as current intake of medicine during the last 12 months had been recorded. Subjects had also been asked to state lifetime prevalence of diseases found by their own physician in 18 categories. Interviewing physicians checked allergic conditions and current medicine intake by inspecting documents stating allergies and medicine supply during the study related house call.

Lifestyle factors and individual exposure to microorganisms from household sources (composting of organic waste in the garden, use of homemade or other compost, organic waste collection in the house, inhalers, soft furnishings) had been determined (Herr *et al.* 2003). This same questionnaire was used in the follow-up in 2002.

Measurement of specific antibodies: In addition the determination of serum concentrations of specific IgG antibodies was offered to all study participants. Those who were reparticipants in the urban area and took part in the blood sampling for the determination of specific IgG antibodies were appointed as “subgroup AB”.

Antigen solutions were prepared from pure cultures of the above-mentioned molds and actinomycetes identified in the air at German compost plants and used for development of an enzyme linked immunosorbent assay (ELISA) as previously reported (Bünger *et al.* 2000).

Briefly: microtiter plates (Maxisorp F96, Nunc, Denmark) were coated with antigens at concentrations from 4 to 20 µg/ml and incubated at 4°C overnight. After washing and blocking, the plates were dried and stored at -20°C until use. For the assay 100 µl serum diluted from 1:400 or 1:800 with PBS was pipetted to each well of the microtiter plates. Controls for nonspecific binding (PBS), as well as a negative serum and the pooled positive serum were tested with each microtiter plate. After incubation for one hour at 37°C the plates were washed thrice, and 100 µl PBS containing 0.1% peroxidase coupled anti human IgG (Organon Teknika) was added. The plates were incubated again for 1 hour at 37°C and washed thrice. Then 100 µl of the substrate buffer containing 0.2% ABTS (2,2-Azino-di-(3-ethyl-benzthiazoline-6-sulfonate)) (Boehringer, Mannheim, Germany) and 0.2% H₂O₂ were

added. The optical density (OD) of each well was measured after an incubation of 30 minutes at 37°C using a microtiter plate reader (Spectra, SLT Instruments, Crailsheim, Germany). Serum concentrations of the study participants were calculated in U/ml from OD-values in relation to the reference values of positive and negative control sera.

Data evaluation: Rates for health parameters were calculated for all participants of the cross sectional study in 1997 and subgroups taking part in follow-up and determination of IgG antibodies against specific microorganisms in serum (subgroup in follow up 2002), not exposed controls and residents of a neighborhood with bioaerosol pollution in outdoor air. (see Table 1). Furthermore all antibody concentrations determined in 2002 were evaluated addressing characteristics of residential area, gender and age. For this analysis of variance of the different types and sums of antibody concentrations was performed using SAS© Version 8.2. Investigated study groups looked upon adjustments were calculated for various variables including age, gender, education, region and atopy.

RESULTS

In 1997 Concentrations of $>10^5$ colony forming units (CFU) of thermophilic actinomycetes, molds, and total bacteria / m³ air had been measured 200 m from the site, dropping to near background concentrations within 300 m. Positive adjusted associations had been observed for residency within 150-200 m from the site versus not exposed controls for some self reported health complaints (e.g. “bronchitis”, “waking up due to coughing”, “coughing on rising or during the day”, “fatigue”). Reports of irritative airway complaints were associated with residency in the area of the highest bioaerosol exposure and period of residency for more than five years (Herr *et al.* 2003).

In 2002 of the 22 of 112 subjects participating in blood sampling had resided near this formerly studied plant at the time of operation. Those were characterized as “follow up, exposed” while the rest (the group in the rural area, where results of measurements of microorganisms in 2002 were equal or beyond background levels, participants of the control groups and those with a period of residency less than 5 years in the exposed urban area) were selected as “follow up, not exposed”.

Table 1. Characteristics and prevalence of health complaints of all participants of the cross sectional study in 1997 and subgroups taking part in follow-up and determination of IgG antibodies against specific microorganisms in serum (subgroup in follow up 2002), not exposed controls and residents of a neighborhood with bioaerosol pollution in outdoor air.

Distance from the emitting site Bioaerosol pollution in residential air	Not exposed controls				Exposed residents			
	--				150-500m			
	Not measured				Up to >10 ⁵ CFU ¹ m ⁻³			
	Total 1997		Subgroup in follow up 2002		Total 1997		Subgroup in follow up 2002	
Studygroup	n=142		n=19		n=214		n=24	
	SS	Yes [%]	SS	Yes [%]	SS	Yes [%]	SS	Yes [%]
Female	142	52.8	19	100	214	59.3	24	95.8
Age >50 ys	142	36.6	19	47.4	214	47.2	24	62.5
Duration of present residency >5 ys	137	70.8	17	88.2	213	72.3	18	94.4
Odor annoyance in the residential area	132	25.8	18	33.3	212	80.2	24	95.8
Odor annoyance: disgusting	37	0.0	6	0.0	162	9.3	23	4.3
Separate collection of organic household waste	136	75.0	19	89.5	212	42.9	24	41.7
Composting in own garden	137	65.7	19	84.2	213	68.5	24	70.8
Occupation at a composting site	136	0.0	19	0.0	201	1.0	23	0.0
Smoking status	132	25.0	19	26.3	192	27.6	24	12.5
Environmental tobacco smoke	111	39.6	15	26.7	172	39.5	21	23.8
Use of inhalers at home	140	7.1	19	15.8	203	11.8	22	9.1
Bedroom equipment ³	142	99.3	19	89.5	213	96.2	24	91.7
Exposure in the workplace ⁴	136	28.7	19	21.1	213	18.3	24	12.5
Home <50m from busy street	142	17.6	19	15.8	214	39.3	24	33.3
Bronchitis	142	26.8	19	15.8	213	33.3	24	50.0
Shortness of breath at rest	137	5.1	19	5.3	206	18.4	24	12.5
Shortness of breath following exertion	136	16.2	19	15.8	208	31.3	24	33.3
Waking up due to coughing	138	25.4	19	15.8	205	41.5	24	54.2
Cough on rising / during the day ⁵	142	19.0	19	15.8	213	35.2	24	37.5
Smarting eyes > 10x/year	136	15.4	19	10.5	208	35.6	21	0.0
Excessive tiredness >5x/year	138	13.0	18	5.6	203	40.4	17	11.8
Shivering	140	13.6	19	5.3	213	19.7	24	12.5
Current intake of medicine	142	41.5			213	56.8		

¹ CFU, colony forming units,

² SS, sample size,

³ Including one of the following: carpet, furs, eiderdown, horsehair or innerspring mattress, furniture made of chipboard,

⁴ Vapours, gases, dusts, heat, cold, dampness,

⁵ Criteria of the World Health Organization for chronic bronchitis

Formerly exposed subjects that had complained of odor annoyance in 1997 (at the time of operation of the plant) were more likely to participate in the follow up 2002. The same applied, if they had complained of cough related complaints in 1997 but not for if “excessive tiredness” had been reported. The latter also applying for subjects that had not been exposed in 1997 and were now participating in the follow up.

The total of IgG antibody concentrations for actinomycetes in the group formerly exposed to residential bioaerosols (“follow up, exposed”) was (min=187U/ml; max=560U/ml; mean=333U/ml; SD=88U/ml; median=323) and in the control group (“follow up, not exposed”, min=128U/ml; max=1,081U/ml; mean =326U/ml; SD=157U/ml median=295U/ml). In the following analysis of variance no association between former exposure to residential bioaerosols in 1997 and the antibodies against actinomycetes and molds could be shown.

This analysis also included age, gender, history of hay fever or other allergy and place of residence in 1997 and did not clear up much of the variance of antibody concentrations ($R^2=0.101$). History of hay fever or other allergies were also not relevant. No difference concerning the region of residency (rural or urban) was found. Women showed a higher titer of total specific IgG concentrations against actinomycetes (mean=338 U/ml; SD=116U/ml) and molds (mean=765U/ml; SD=561U/ml).

In further analyses differences in antibody concentrations being determined in 2002 were observed for gender, smoking status and those using inhalers. Differences in antibody concentrations against three of four *aspergillus* species remained significant after analysis of variance including gender and exposition in 1997 for gender only.

DISCUSSION

Considering the characteristics of the study group (Table 1) it can be deduced that that participants of the follow up undergoing blood sampling were to a greater extent women and older than in the whole study group. Also the formerly odor annoyed tended to participate more readily in the follow-up. These factors might be considered to bias reported health in the follow-up. It cannot be deduced from this that the study group of antibody sampling was biased in such a way by this that not differences were found between formerly exposed and subjects without bioaerosols exposure.

The reported health complaints also show a difference between the whole study group participating in 1997 and the follow up sample of 2002. This could imply that a risk group concerning health impairment due to bioaerosols pollution had

undergone antibody determination five years after closure of the plant. As far as antibody formation to the specific microorganisms is concerned this group could not be identified as being at special risk compared to the other subjects in this study. It has to be considered in this context though that persistence of these specific antibody concentrations in human blood, is not known.

The higher levels of single antibody concentrations for women cannot be cleared up though. Further studies should take include parameters like the duration of staying at home where possibly women are exposed more likely because of social, occupational and hygienic habits, which might also be a reason for longer exposition to the measured outdoor concentrations in the studied area in 1997. Indoor storage of organic waste was found to be a risk factor for skin-related health complaints in another evaluation (Herr *et al.* 2004) and therefore should be included in further investigations.

The ubiquitous spread of molds might explain comparable titers found in antibody concentrations against *Aspergillus species* in subjects formerly exposed to residential subjects without exposure. Results also indicate high variances in specific IgG antibody levels against actinomycetes. No reference values have been established for these antibodies to date.

CONCLUSIONS AND IMPLICATIONS

Five years after closing down of a composting plant causing bioaerosol pollution of the adjacent residential area no continuing differences of specific antibodies between the study groups were assessable in the biomonitoring. The high variances in specific antibody levels need to be cleared up in further investigations that should include measurements of microorganisms in the residential air.

ACKNOWLEDGMENTS

The study was coordinated by the “The Institute for Applied Microbiology of the Justus-Liebig-University Giessen” and was supported by a governmental funding source, the “German Federal Ministry of Education and Research, Jülich Research Centre, Berlin”. Grant No. 0330279 (full text study report (German) for download at <http://www.bacterium.de>).

The excellent technical assistance of Martina Lange, Anke Seeckts, and Jutta Utermöhle in the determination of specific IgG antibodies is gratefully acknowledged.

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Chapter
Assessment IV – Mycology (continued)

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MYCOLOGY

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ABSTRACT

Measurement methods for the assessment of fungal contaminants in indoor environments have traditionally been based on culture and microscopy. Immunoassay, enzyme assay, and molecular biology-based methods provide enhanced detection capabilities. However, all methods have limitations. This session represented papers reporting new applications and developing protocols for fungal monitoring in indoor environments.

INDEX TERMS: Mycology, monitoring, traditional methods, enhanced techniques

INTRODUCTION

Fungal contaminants in indoor environments have been associated with adverse human health effects. Assessment of the risk of exposure of building occupants includes measurement of the concentration and population of fungi present on surfaces and in the air. Measurement methods for the assessment of fungal contaminants in indoor environments have traditionally been based on culture and microscopy. These methods provide enumeration and identification of culturable and total fungal structures, respectively, but both methods have limitations. Recently developed methods including immunological assay and molecular biology techniques can enhance detection, but these methods also have limitations. Presentations in this session discuss the traditional and developing technologies for the assessment of fungal contaminants in indoor environments. The following is an overview of each method.

TRADITIONAL METHODS

Culture: The cultivation of viable fungi is a traditional method for detection and identification. Success of culture-based analysis depends on the physiological state of the organism and the selection of appropriate nutrient media, incubation tem-

perature, relative humidity, and other physical conditions. While many fungi are resistant to environmental stresses, sampling and handling conditions may decrease viability resulting in poor culturability. Therefore, storage and shipment of sample for culture-based assay must be considered.

There are numerous media formulations that are used for the isolation of molds and yeasts, but malt extract agar with incubation at room temperature is often used for routine isolation. However, xerophilic fungi grow in a low moisture environment so they may not be detected unless a medium amended with glycerol (e.g., dichloran glycerol, DG18) is used to enhance their growth in culture. Other fungi will not grow in the presence of other genera. For example, *Stachybotrys*, often will not grow in culture when high concentrations of *Aspergillus* and/or *Penicillium* are present on a malt extract agar plate. The use of a growth medium with cellulose as the only carbon source increases the success of isolating *Stachybotrys* and the use of multiple platings on different growth media is done to encompass the range of fungi anticipated in a sample.

Sufficient incubation time and correct incubation temperature is needed to permit the sporulation and growth of visible colonies. Culture requires 3-5 days to produce visible colonies of mesophilic fungi with characteristic colors and textures. Additional time is often required for sub-culturing onto a variety of media to stimulate formation of characteristic structures and/or exudates for speciation of many genera. Mesophilic species are cultured at room temperature. Thermophilic fungi with optimal growth temperatures $\geq 40^{\circ}\text{C}$ are isolated and discriminated from mesophiles by using increased incubation temperatures.

Overcrowding on the agar surface due to high concentrations of organisms and inhibition by some species may result in the lack of growth and reporting of "false negatives." Overgrowth by rapidly spreading fungi (e.g., *Rhizopus* and *Trichoderma*) may obscure the detection of slower growing colonies. The addition of rose bengal or other dye is used to decrease the spread of rapidly growing organisms and permit the isolation of slower growing fungi.

Identification of fungal genera and species is limited by the expertise of the analyst and it is subjective, often resulting in widely varying inter- and intra-laboratory identifications. Stocks of standard strains, numerous reference texts, and a photographic library for comparison are useful in maintaining the proficiency of fungal analysts.

Microscopy: Viewing of fungal structures using light microscopy with a 400-600x magnification is used to enumerate and identify total (viable and nonviable) microorganisms. Identification of most fungal spores is reported to the genus level, but without specific staining or other enhancements, microscopy can only

differentiate a few fungi to species level based on classical structures. Analysis with a stereomicroscope or low magnification light microscopy can be used to directly inspect samples for fungal structures. Air and surface samples can be rapidly assessed for the presence of fungal spores, hyphae, and other structures, but this technique is hampered by the presence of abiotic and biotic particulate. Overloading of the collection/viewing surface by increased sampling times can result in debris obscuring the presence of fungal material. Similar to culture, identification of fungal structures by microscopy is limited by the expertise of the analyst and is subjective. Reference texts and a photographic library are needed to maintain proficiency of the analysts.

ENHANCED METHODS

Immunoassay and Enzyme Assay: Several advanced techniques have recently been applied to environmental samples for the enhanced detection, enumeration, and identification of fungi. Immunoassay relies on specific antigen/antibody reactions to signal the presence of the organism of interest. The use of polyclonal and monoclonal antibodies is useful in providing rapid indications fungal antigen in a sample. Specificity is often a concern as many fungal genera and species within genera share common epitopes. Assays that rely on enzymatic activity can also provide a rapid means to detect the presence of microorganisms. However, many enzymes are common among fungi and other microorganisms, so specificity can also be a problem with this technique. Interferences from biotic and abiotic substances also are a concern with immunoassay and enzyme assays.

Molecular Biology: The use of molecular biology-based methods enhances the detection and identification of environmental fungi. Polymerase chain reaction (PCR) is a technique in which specific gene sequences are amplified to increase the ability to detect their presence in a sample. Until recently, PCR protocols were accompanied by gel electrophoresis to visualize bands of nucleic acid representing the organism of interest. While this provided data on the presence of target organisms, it did not adequately enumerate the organisms. Quantitative PCR (QPCR) incorporates real-time amplification with fluorescently labeled probes to provide a rapid, reliable detection and enumeration of the number of gene templates present in a sample. PCR and QPCR techniques require knowledge of specific gene sequences to provide specificity and discriminate the target organism from closely related organisms. Quantitative PCR also requires preparation of standards and performance of quality assurance trials to maintain accuracy and precision of the method. While this is a sensitive technique permitting the detection of a single organism, interference by other nucleic acids and organic and inorganic compounds is a problem that often requires extensive sample preparation. The incorporation of internal positive controls alerts the analyst that interference is present,

necessitating additional sample manipulation and analysis. Restriction fragment length polymorphism (RFLP) is a molecular biology-based technique in which gene sequence fragments produced by treatment with specific endonucleases are used to discriminate closely related organisms. Profiles of populations can be used for comparison. However, molecular biology-based methods do not provide data on viability as they detect the presence, not the physiological state of the organisms.

Traditional and enhanced methods provide data to detect and enumerate fungi in environmental samples, but all methods have limitations. The following papers are among those presented at the Assessment IV – Mycology session utilizing culture, microscopy, or enhance techniques. They represent new applications and developing protocols for monitoring in indoor environments.

CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES AGAINST *ASPERGILLUS VERSICOLOR* AND OTHER FUNGI AND THEIR IMPLICATIONS FOR THE DEVELOPMENT OF ANTIBODY-BASED MONITORING TECHNIQUES FOR FUNGI

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ABSTRACT

In order to improve current culture-based monitoring techniques for *Aspergillus versicolor* and other fungi in general, our laboratory is developing species-specific monoclonal antibody (mAb)-based immunoassay techniques. In this study we attempted to enhance the production of mAbs specific for *A. versicolor* by immunizing mice with pre-formed immune complexes to block epitopes of spores known to be cross-reactive with other fungi. We found that immunization with immune complexes did not enhance the production of *A. versicolor*-specific mAbs. While the antibody reactivity to related *Aspergillus* and *Penicillium* species was reduced, the response to other fungi was enhanced. The patterns of cross-reactivity among the 50 tested fungal species not only demonstrate that phylogenetically distant fungi share common epitopes but also that different fungi express a given cross-reactive epitope in variable amounts on a per spore basis.

INDEX TERMS: Monoclonal antibody, cross-reactivity, ELISA, *Aspergillus versicolor*, monitoring techniques

INTRODUCTION

Many fungi have been associated with a variety of adverse health effects (Fung, Hughson, 2003; Kolstad *et al.* 2002) in indoor environments and, therefore, need to be monitored in order to document exposures and complement rational patient management. Current monitoring techniques are either based on the cultivation of environmental samples or the biochemical detection of marker molecules such as (1-3)- β -D-glucan, ergosterol or extracellular polysaccharides (Burge, 2002;

Pasanen,, 2001). While these techniques have been useful on a case-by-case basis, they have failed to establish standardized reference techniques for ecological and exposure monitoring purposes. Some of the main problems associated with these techniques are diagnostic challenges due to differential culturability and relative growth potential of different fungi or the introduction of ambiguities in quantitative results due to the differential expression of marker molecules by different fungi on a per spore or unit of biomass basis.

Our approach for the development of objective monitoring techniques for airborne fungi in indoor environments is based on the detection of fungi with enzyme-linked immunosorbent assays (ELISA) using species-specific monoclonal antibodies (mAbs) or phage display-derived peptide reagents. Although antibody-based techniques are being increasingly used for environmental monitoring of chemical contaminants (van Emon, 2001) and for the presence of fungi in food and feed products (Li *et al.* 2000), their use for the detection of fungi in environmental samples has so far been limited. Two of the major problems associated with the application of immunoassays in fungal research are the extensive cross-reactivity of antibodies with a variety of related and non-related fungi and the expression of shared epitopes at differing amounts by each of the cross-reacting fungi on a per spore basis. The analytical implications of cross-reactive antibodies are such that a given ELISA result cannot be translated into actual spore numbers or biomass units for a particular target fungus. This is because identical ELISA results of two samples may be due to the presence of two entirely different mixtures of cross-reactive fungi which happen to express the same total number of antibody epitopes per sample. In order to address these problems in exposure assessment, we are investigating different methods to improve the specificity of monitoring reagents.

Aspergillus versicolor is not only a commonly occurring fungal species in the indoor air spora (Nielsen, 2003; Shelton *et al.* 2002) but it has also been implicated in a variety of adverse health effects (Hodgson *et al.* 1998). In this study we used *A. versicolor* as a model fungus to examine the potential of immune complex immunization for the production of species-specific mAbs that may be of benefit for the accurate spatial and temporal monitoring of the fungus. During immune complex immunization, mice are immunized with the antigen complexed with pre-formed cross-reactive mAbs. The rationale for this approach is that immune complex immunizations have been reported to shift the immune response toward weakly antigenic sites while at the same time tolerizing against immunodominant epitopes (Benkirane *et al.* 1985; Thalhamer, Freund, 1985). They have also been suggested to increase the diversity and the degree of affinity maturation of the resulting antigen-reactive B cells repertoire (Nie *et al.* 1997). Such a directed shift in the immune

response may increase the chances of isolating species-specific mAbs against highly complex antigens such as fungal spores.

In this study, we immunized mice with immune complexes and produced mAbs against spores of *A. versicolor*. Each mAb was then tested against spores of 16 *Aspergillus* species 14 *Penicillium* species and 20 other fungi commonly found in indoor environments. The reactivity of the five newly produced mAbs was also compared to that of a previously reported mAb that was produced against spores rather than immune complexes.

METHODS

Cultivation of fungi and antigen preparation: Fungi were obtained from the American Type Culture Collection and the National Center for Agricultural Utilization Research or isolated from indoor air samples. Isolated fungi were identified based on spore and culture morphology using standard mycological reference literature. All fungi were grown on malt extract agar (2% dextrose, 0.1% peptone, 2% malt extract, 2% agar; Difco, Becton Dickinson, Sparks, MD) plates for 10 days at room temperature. Spores were collected inside a class II biological safety cabinet from air-dried cultures by applying 1 g of glass beads (0.45 – 0.5 Ø, Braun-Melsungen, Germany) per Petri dish. The lid was put back in place and the plates were gently shaken back and forth to allow the beads to roll across the spore-covered agar. We found that following this procedure ample spores were attached to the beads. Beads were poured into a 50 ml tube and spores were suspended in either phosphate buffered saline (PBS, 10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, pH 7.4; Sigma, St. Louis, MO) for antigen preparation or carbonate coating buffer (60 mM sodium carbonate, 140 mM sodium bicarbonate, pH 9.6) for enzyme-linked immunosorbent assay (ELISA) analysis of mAb reactivities. The elution process was enhanced by briefly shaking the tube a few times. Spores were then separated from the beads by decanting the spore suspension after the beads had settled out.

Production of mAbs: The particulate fraction of homogenized spores of *A. versicolor* was incubated for 1h at 37 °C with tissue culture supernatant of either mAb 2B8 (IgG₃) or a mixture of mAbs 4D2 (IgG_{2a}/IgM) and 2C6 (IgG_{2a}/IgM). These mAbs were previously produced against *Penicillium chrysogenum* and shown to be highly cross-reactive with spores of *A. versicolor* (Schmechel *et al.* 2001). Two groups containing two female BALB/c mice were immunized six times intraperitoneally with one of the two different immune complexes. MAbs were produced according to standard technology (Harlow, Lane, 1988). Hybridomas were screened by solid phase indirect ELISA using spores of *A. versicolor*. Positive cell lines were cloned twice by limiting dilution and aliquots were stored in liquid nitrogen.

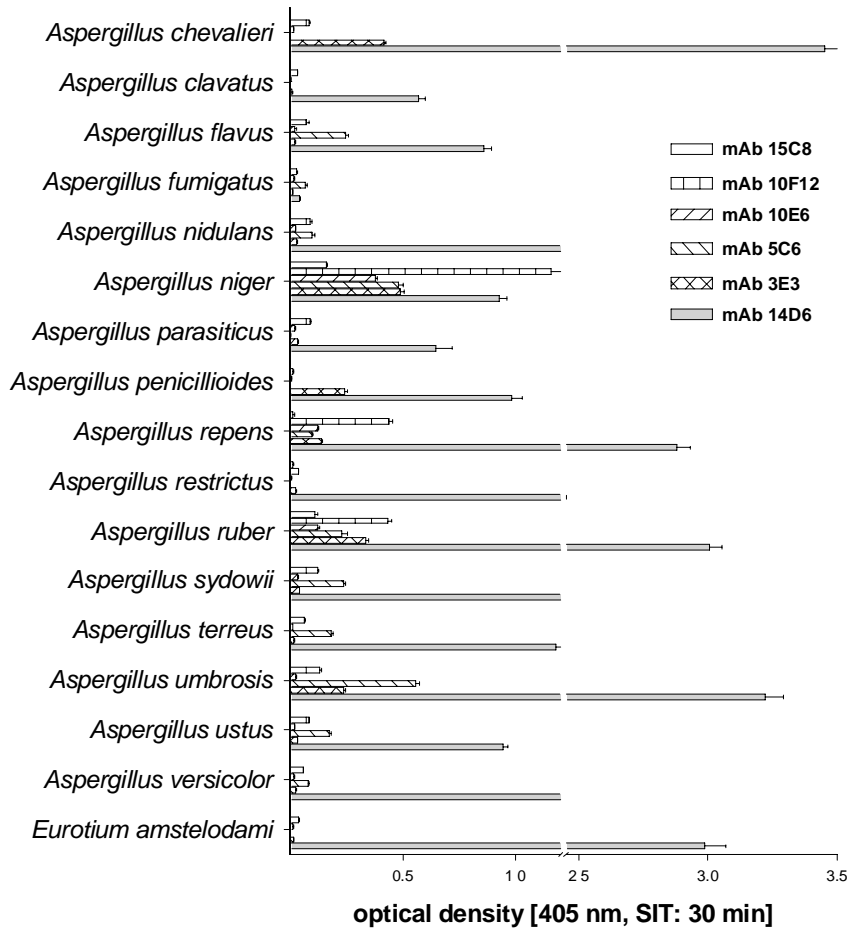
Cross-reactivity ELISA: Spores of 50 fungi were each incubated in ELISA wells overnight at room temperature (RT) in carbonate coating buffer, pH 9.6. Spores were coated at a concentration of 100,000 spores per ELISA well except *Alternaria alternata* (30,700 spores/well), *Aspergillus umbrosus* (37,500), *Aureobasidium pullulans* (24,750), *Epicoccum nigrum* (5,750), *Fusarium oxysporum* (57,250), *Geotrichum candidum* (18,000), *Stachybotrys cylindrospora* (25,500) and *Ulocladium chartarum* (37,500) were coated with fewer spores because of poor sporulation. MAb reactivity was determined with a previously described alkaline phosphatase-based indirect ELISA (Schmechel *et al.* 2002).

RESULTS

A total of 12 mAbs were produced. Based on their cross-reactivity patterns these could be grouped into 5 different groups. Figures 1-3 show the reactivities of one characteristic member of each group. MAb 15C8 represents 4 similar mAbs. Two mAbs (10E6 and 10F12) each represent three mAbs and the groups of mAbs 3E3 and 5C6 only contained a single mAb. Each column represents the average of 3 replicate ELISA well optical densities (OD) and each error bar represents the corresponding standard error of the mean. Fungal species are listed on the y-axis and the corresponding ODs which were determined after a substrate incubation time (SIT) of 30 min are shown on the x-axis. No *A. versicolor*-specific mAbs were produced. Figure 1 presents the mAb cross-reactivities for 16 *Aspergillus* and one related *Eurotium* teleomorph species, Figure 2 shows the results for 14 *Penicillium* species and Figure 3 shows the results for 19 other fungi commonly found in indoor environments. In addition, each graph also shows for comparison the cross-reactivities of mAb 14D6 which was previously produced against spores rather than immune complexes of *A. versicolor* (Schmechel *et al.* 2002).

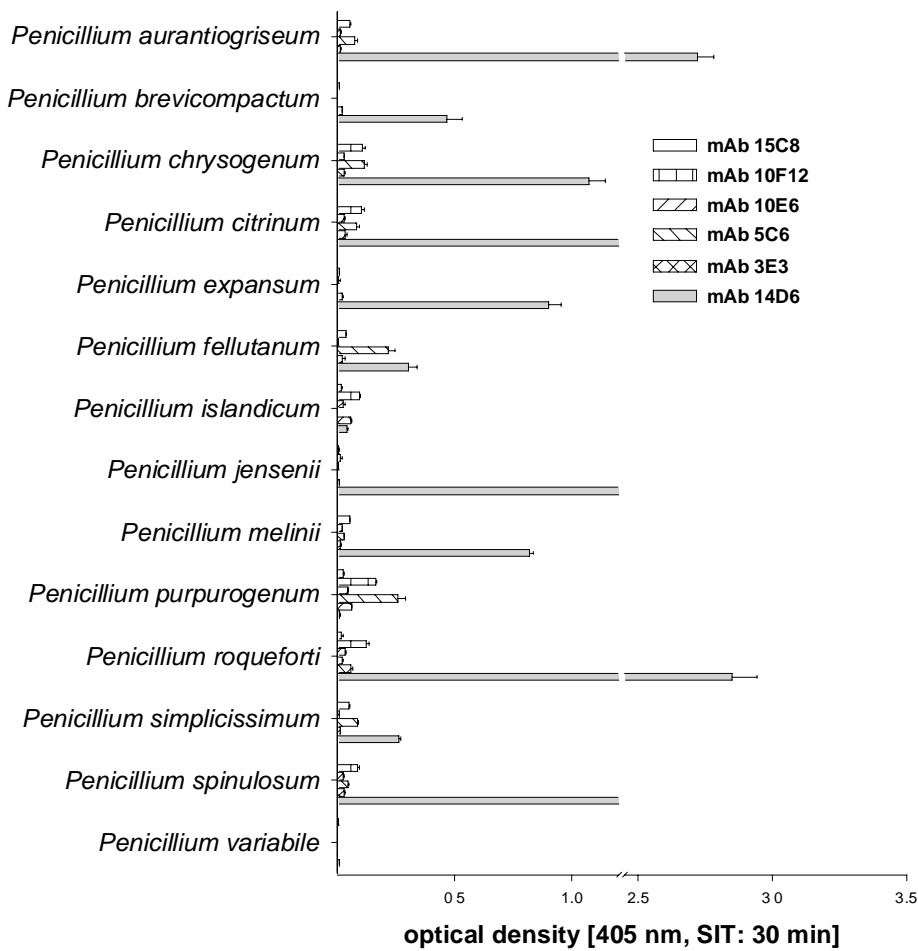
The cross-reactivity patterns of the five newly produced mAbs show that immune complex immunization resulted in a generalized suppression of the immune response to most *Aspergillus* (Figure 1) and *Penicillium* species (Figure 2) when compared to mAb 14D6 which was produced against spores rather than immune complexes. While most of the ODs for mAb 14D6 were between 1 and 2.5, the ODs for all other mAbs were less than 0.25. However, immune complex immunization resulted in a marked increase in the cross-reactivities of the mAbs with other fungal species when compared to immunizing with spores only (Figure 3). Dematiaceous fungi (i.e. *Alternaria alternata*, *Epicoccum nigrum*, *Memmoniella echinata*, *Myrothecium verrucaria*, *Stachybotrys spp.* and *Ulocladium chartarum*) showed considerable cross-reactivity.

Figure 1.



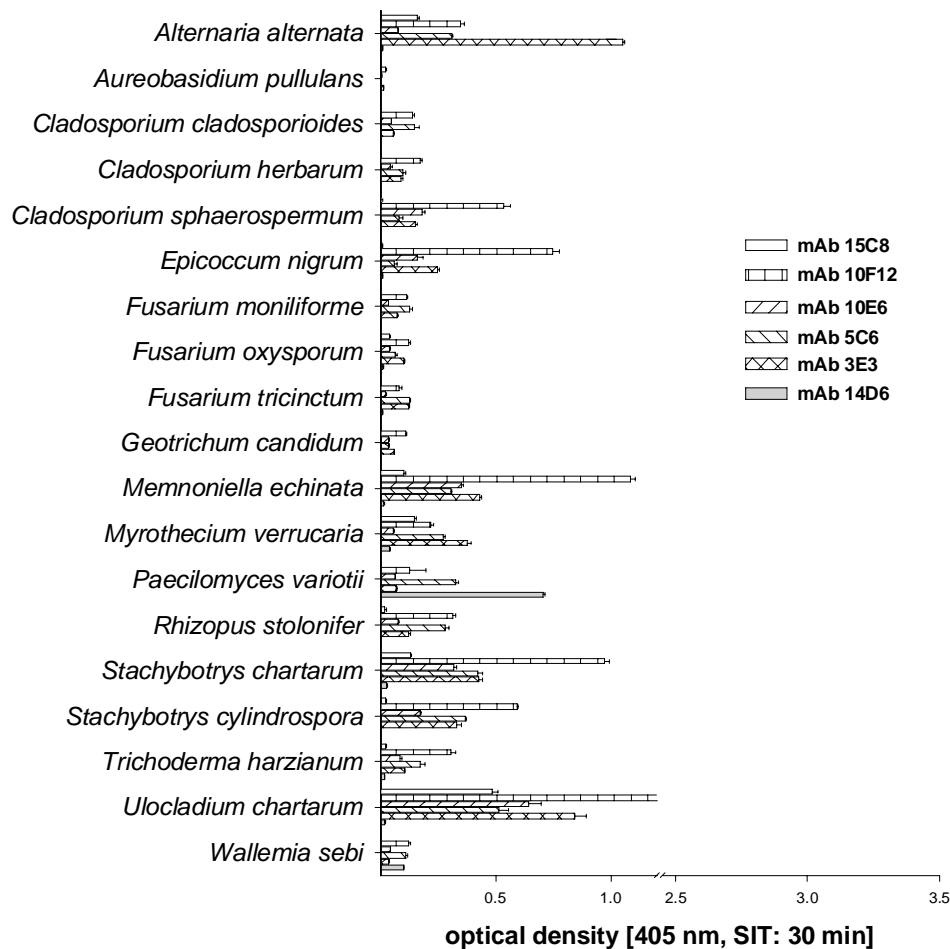
Monoclonal antibody reactivity with 16 *Aspergillus* and 1 *Eurotium* species. MAb 14D6 was produced against the particulate fraction of spores of *A. versicolor* and all other mAbs were produced against immune complexes composed of the particulate fraction of *A. versicolor* and antigen-reactive mAbs.

Figure 2.



Monoclonal antibody reactivity with 14 *Penicillium* species. Mab 14D6 was produced against the particulate fraction of spores of *A. versicolor* and all other mAbs were produced against immune complexes composed of the particulate fraction of *A. versicolor* and antigen-reactive mAbs.

Figure 3.



Monoclonal antibody reactivity with 19 fungal species commonly found in indoor environments. MAb 14D6 was produced against the particulate fraction of spores of *A. versicolor* and all other mAbs were produced against immune complexes composed of the particulate fraction of *A. versicolor* and antigen-reactive mAbs

DISCUSSION

The reduced cross-reactivity of all five mAbs with related *Aspergillus* and *Penicillium* species in combination with the failure to generate any species-specific mAbs suggests that the immunization of immune complexes results in a general state of immune suppression toward the target fungus as well as related fungi and does not increase the immunogenicity of target fungus-specific epitopes. Furthermore, the increased cross-reactivity between many dematiaceous fungi and several *Aspergillus*

and *Penicillium* species suggest that phylogenetically distant fungi share numerous common antibody binding sites and that the immunogenicity of the shared epitopes is more pronounced after the suppression of the genus-specific immune response. The highly variable reactivity of a given mAb with normalized amounts of spores of different fungi suggests that shared epitopes may be expressed at substantially different amounts on a per spore or unit biomass basis by different fungi. In related research, immune complex immunizations also failed to generate species-specific mAbs to *Stachybotrys chartarum* (Schmechel *et al.* 2003), *Penicillium brevicompactum* and *P. chrysogenum*.

CONCLUSIONS AND IMPLICATIONS

We conclude that immune complex immunization did not stimulate the production of species-specific mAbs. In order to generate such reagents, other tolerization methods such as neonatal tolerance or subtractive immunization using cyclophosphamide need to be investigated. Alternatively, other techniques such as synthetic or phage display of combinatorial antibody or peptide library techniques should be examined for their potential to provide desired detection reagents. We are currently using random peptide phage libraries to generate species-specific reagents to *A. versicolor* and other fungi.

ACKNOWLEDGEMENT

This study was funded by internal grant money from the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention.

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CHARACTERIZATION OF FUNGAL FLORA FROM MOISTURE-DAMAGED BUILDING MATERIAL BY rDNA SEQUENCING AND CULTURE

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ABSTRACT

In this study, rDNA sequencing and culture were used to characterize the fungal flora in moisture-damaged building material. This was accomplished by creating and sequencing a clone library of ITS area of rDNA from total DNA extracted from studied material. The ability of two-enzyme RFLP analysis to screen for different inserts was tested. The sequencing result was compared with the result obtained by traditional culture methods. Of the 31 sequence types obtained, at least 35% were chimeric. RFLP separated 74% of the sequence types. Eight sequences were identified to species or genus level, and sequences from 12 to 19 unknown or as yet unsequenced fungal species were found. Six fungal genera and non-sporulating isolates were found by cultivation. Only one genus was detected by both of the methods. The profound differences in the two methods complicated the comparison.

INDEX TERMS: Fungal diversity, moisture damage, rDNA, internal transcribed spacer, RFLP

INTRODUCTION

According to current opinion, several different symptoms occurring in residents and workers of water-damaged buildings are likely due to microbial growth in building materials. Dampness, which promotes microbial growth, has been shown to correlate with symptoms (Bornehag *et al.* 2001). The adverse effect to health is thought to be dependent on the species diversity and varying metabolites produced by different microbial groups (Samson *et al.* 1994). Several potent harmful compounds produced by fungi (i.e. mycotoxins) have been identified, many of which have also been detected in water damaged building materials (Tuomi *et al.* 2000).

The traditional methods to count and identify fungi in air and in material samples rely on cultivation of the samples, and on identification of spores and other characteristic morphological structures by direct microscopy. Culture-based methods are insufficient in providing a reliable picture of the total microbial flora present. “The great plate count anomaly”, a phenomenon long known to affect the number and diversity of microorganisms isolated in plate culture, and the difficulty of taxonomic identification of some fungal morphotypes are the major factors contributing to this problem. Therefore, new, more accurate identification methods have been developed. DNA-based methods are increasingly used due to their wide applicability and high specificity. These benefits, and the speed of identification are valuable, especially in many clinical studies (Iwen *et al.* 2002). Most studies of indoor microbial populations have focused on specific fungal or bacterial groups by using group specific probe or PCR systems (Rintala *et al.* 2002; Cruz-Perez *et al.* 2001; Haugland *et al.* 1999).

In this study, a universal approach towards detecting all or most of the fungal species in a building material sample was taken by creating a clone library that represented the evolutionarily variable Internal Transcribed Spacer (ITS) -area of genomic ribosomal DNA of fungal species in the sample. The procedure described here has not been applied in indoor studies before, yet it has been used to describe fungal flora in other environments, e.g. plant tissue and rhizosphere (Borneman, Hartin, 2000; Guo *et al.* 2001) as well as in several 16S rDNA gene studies focusing on prokaryotic diversity (Rappé, Giovannoni 2003). The basic method can thus be applied to virtually any starting material and microbial group of interest, where culturing is seen impossible or insufficient.

METHODS

DNA extraction: Total DNA was extracted from 1 g of water-damaged wood material from the board lining in an outer wall. The extraction method used was a modification of previously described protocol (Rintala *et al.* 2001) and consisted of mechanical disruption and chloroform extraction, followed by additional purification by Wizard Clean Up-column (Promega). Disruption was done by 30 min vortexing with sterile sand in sample buffer (described in Kuske *et al.* 1998).

PCR amplification and DNA cloning: The ITS area, comprising conserved 5.8S rDNA gene and variable flanking ITS-1 and -2 regions was PCR-amplified with ITS1 and ITS4 primers (White *et al.* 1990). The reaction and thermal cycling conditions have been previously described by Hendolin *et al.* (2000). The reaction mixture contained DMSO and betaine as supplements to stabilize the DNA polymerase and weaken the relative strength of GC-base pairing vs. AT-pairing. High PCR cycle number (38) was used to obtain maximum amount of product for

cloning. Water treated as sample material was used as negative control. The yield of PCR was estimated by standard gel electrophoresis and ethidium bromide staining. 80 ng of purified PCR product was cloned into an *E. coli* plasmid library with commercial T/A cloning kit according to the manufacturers instructions.

DNA sequencing and RFLP analysis of inserts: Inserts of the library were re-amplified with ITS1/4 primer pair and analyzed for RFLP variability by separate digestion with two restriction endonucleases. 500 ng of each of the re-amplified ITS inserts from the library was digested with TaqI and HinfI restriction endonucleases. Digestion fragments were separated in EtBr-stained agarose gel and classified according to the digestion patterns. One or both strands of all PCR products were sequenced using ITS4 or 1 as primers in cycle sequencing reactions. Identical sequences were grouped and different sequence types were aligned with each other as well as with the sequences in public DNA databases.

Phylogenetic analyses: Multiple sequence alignments were committed using ClustalW, and homology searches using Fasta3 program offered by the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI, Cambridge, UK). Plant and fungal DNA sequence databases of EMBL-EBI were used as target in homology searches. When high sequence similarities were found, identity of the sequences was determined from the closest relatives found in the database. When only low similarity values were seen, ITS-1 and -2 -areas were localized and analyzed also separately.

Culture methods: The sample material was finely ground using sterile knife and the microbial cells were separated from the material by shaking and sonication in sample buffer (42,5 mg/l KH₂PO₄; 250 mg/l MgSO₄ x 7H₂O; 8 mg/l NaOH; 0,02% Tween 80). Fungi were cultured on malt extract agar (made of 2% malt extract [Biokar Diagnostics, Beauvais, France] and Bacto-agar [Becton Dickinson and Company, Le Pont de Claix, France]) and dichloran glycerol agar (DG18, Oxoid Ltd., Basingstoke, England) plates in room temperature. Morphological identification and counting of the colonies was done after 7 days of incubation.

RESULTS

RFLP analysis and sequencing: Sequencing of 192 clones produced 167 sequences of 31 different types. Two sequence types represented the majority (68%) of the library, 19 were present as a single clone, which indicates that the sample library was too small to find majority of different sequence types in the material. Digested PCR products divided into 23 different RFLP types. One RFLP class was common to five, two classes were common to two, and the remaining 21 represented a single sequence type. The sequences in same class differed by 1-25%.

Four sequences had 399% similarity, five had 97-99% and the remainder had 74-97% similarity with database sequences when the whole ITS area was aligned (Table 1). The alignment of ITS-1 and -2 areas as separate blocks with each other (data not shown), as well as with the database sequences (Table 1) showed behavior typical to chimeric molecules in case of several sequences. Some sequence types with clearly different overall sequence had identical ITS-1 or -2 areas. The two ITS blocks in one sequence could also have high similarities with taxonomically far relatives in the database when the overall similarity with known sequences was low. In some cases, the natural parent molecules could be found for chimeric daughter sequences. In cases where the similarities to database sequences were low, the origin or chimerism could not be reliably determined. From the nature of ITS area, and from what is known about PCR recombination and formation of chimeras (Wang, Wang, 1996), it was supposed that the most probable spot for chimera junction was the conserved 5.8S gene between the flanking ITS blocks. Thus by excising the gene and analyzing the ITS blocks separately, more species were identified from the material. The following taxons were identified as present in the sample due to sequence similarity of at least either of the ITS blocks with database sequences: the species *Graphium rubrum*, *Phialophora lagerbergii*, *Hyaloscypha aureliella* and *Phomopsis quercella*, the genera *Exophiala*, and probably *Cladosporium*, *Bensingtonia* and *Phoma* or close relatives, and the order *Helotiales*. Sequences from 12 to 19 unknown or yet unsequenced fungal species were also found. Only one basidiomycetous sequence was found (*Bensingtonia*), the rest belonged to the *Ascomycota*. One identified sequence belonged to green alga *Chlorella* (Table 1).

Culture methods: By traditional cultivation and morphological identification the genera *Penicillium*, *Acremonium*, *Rhinochrysiella*, *Exophiala* and *Cladosporium*, and Sphaeropsidales-group fungi, yeast and non-sporulating isolates were found.

Table 1. Alignment of the library sequences with EMBL Plant and Fungi databases. The variable ITS segments (ITS1 and 2) were aligned separately as well as together with the conserved 5.8S gene.

Clone id.	FASTA-alignment				Chim ^a	
	whole ITS-area	ITS-1	ITS-2			
#31	<i>Pseudogymnoascus roseus</i> AF062819 93%	<i>Geomyces pannorum</i> AF015789	<i>G. pannorum</i> AF307760	86%	96%	N
#32	<i>G. rubrum</i> AF198245	<i>G. rubrum</i> AF198245	<i>Hyaloscypha aureliella</i> U57495	92%	98%	P
#38	<i>Chlorella</i> sp. AF479758	<i>Chlorella</i> sp. AF479758	<i>Chlorella</i> sp. AF479758	99%	100%	N
#38	<i>G. rubrum</i> AF198245	<i>Graphium rubrum</i> AF198245	<i>Axenic ectomycorrhizal isolate</i> AJ430406	100%	99%	P
#44	<i>Antarctomyces psychrotrophicus</i> AJ133431	Unidentified ascomycota AJ301722	<i>Mycosphaerella brassicola</i> AF297223	89%	74%	-
#47	<i>Phialophora lagerbergii</i> AF083197	<i>Phialophora lagerbergii</i> AF083197	<i>G. pannorum</i> AF307760	99%	95%	P
#48	<i>Chlorella ellipsoidea</i> D13340	no match	<i>C. ellipsoidea</i> D13340		67%	N
#56	<i>Umbilicaria americana</i> AF096218	<i>Umbilicaria leiocarpa</i> AF096211	<i>Melanella hepaticon</i> AF141369	84%	74%	-
#60	<i>Bensingtonia yamatoana</i> AB038079	<i>B. yamatoana</i> AB038079	<i>B. yamatoana</i> AB038079	96%	98%	N
#61	<i>Salal root associated fungus</i> AF149071	<i>H. aureliella</i> U57495	<i>H. aureliella</i> U57495	99%	96%	N
#63	<i>Capronia acutiseta</i> AF050241	<i>Ectomycorrhizal ascomycete</i> AF373060	<i>Capronia munkii</i> AF050248	83%	76%	-
#64	<i>Phialophora</i> sp. AF083200	Unidentified ascomycota AJ301722	<i>Axenic ectomycorrhizal isolate</i> (Helotiales) AJ430406	88%	99%	P
#76	<i>Solenopezia solenia</i> U57991	<i>Rhizosphaera</i> sp. AF462430	<i>Ericoid mycorrhizal</i> sp. AY046399	80%	65%	-
#79	<i>Phoma</i> sp. AF218789	<i>Exophiala</i> sp. AJ301732	<i>Phoma</i> sp. AF218789	100%	96%	P
#81	<i>Phoma</i> sp. AF218789	<i>Umbilicaria lyngei</i> AF297669	<i>Phoma</i> sp. AF218789	79%	97%	P

Table 1. Continued

Clone id.	FASTA-alignment				Chim ^a		
	whole ITS-area	ITS-1	ITS-2				
#85	<i>Phialophora</i> sp. AF083200	91%	<i>Axenic ectomycorrhizal isolate (Helotiales)</i> AJ430406	98%	<i>Geomyces asperulatus</i> AJ390390	96%	P
#87	<i>Pseudocercospora basiramifera</i> AF309595	82%	<i>Capnobotryella</i> sp. AJ301706	95%	<i>Mycosphaerella fijiensis</i> AF297225	76%	-
#90	<i>G. asperulatus</i> AJ390390	92%	<i>G. pannorum</i> AF015789	84%	<i>G. pannorum</i> AF307760	95%	-
#96	<i>Phoma</i> sp. AF218789	96%	<i>Phoma</i> sp. AF218789	96%	<i>Axenic...</i> AJ430406	99%	P
#109	<i>Phialophora botulisporea</i> AF083198	93%	<i>Exophiala</i> sp. AJ301704	100%	<i>Phialophora botulisporea</i> AF083198	96%	P
#115	<i>G. rubrum</i> AF198245	100%	<i>G. rubrum</i> AF198245	100%	<i>Phialophora</i> sp. AF083204	100%	N
#116	<i>Phomopsis quercella</i> AJ293878	92%	<i>Unid. ascomycota</i> AJ301722	90%	<i>P. quercella</i> AJ293878	99%	P
#119	<i>Phoma</i> sp. AF218789	98%	<i>Phoma</i> sp. AF218789	97%	<i>Phoma</i> sp. AF218789	97%	N
#121	<i>P. quercella</i> AJ293878	99%	<i>P. quercella</i> AJ293878	100%	<i>P. quercella</i> AJ293878	100%	N
#124	<i>Iceman fungal clone</i> X88771	98%	<i>Iceman fungal clone</i> X88771	98%	<i>Iceman fungal clone</i> X88771	98%	N
#125	<i>Axenic...</i> AJ430406	99%	<i>Axenic...</i> AJ430406	98%	<i>Axenic...</i> AJ430406	99%	N
#137	<i>Unid. ascomycota</i> sp. AJ279469	87%	<i>Exophiala</i> sp. AJ301704	100%	<i>Unid. ascomycota</i> AJ279469	84%	-
#140	<i>Leotiales</i> sp. AY011014	86%	<i>U. americana</i> AF096218	81%	<i>Salal root associated fungus</i> AF300755	83%	-
#145	<i>Cladosporium elatum</i> AF393699	97%	<i>Coniothyrium</i> sp. AJ301702	99%	<i>C. elatum</i> AF393699	94%	N
#146	<i>A. psychrotrophicus</i> AJ133431	84%	<i>Unid. ascomycota</i> AJ301722	89%	<i>C. elatum</i> AF393699	94%	P

The chimerism of the sequences was determined from the behaviour the of the ITS segments in database and multiple sequence alignments. P: probably chimeric; N: not probably chimeric; -: chimerism could not be determined.

DISCUSSION AND CONCLUSIONS

Eight fungal species or representatives of higher groups were identified by means of sequencing. The culture method detected six genera or higher group and unidentifiable sterile mycelia and yeast cells. The genus *Exophiala* was found by both methods. *Penicillium* or *Acremonium* were seen only in culture, and no sequences similar with these genera were found in the clone library. The genus *Phoma* found by sequencing may have been classified under Sphaeropsidales group in morphological identification. It is also possible that cultured genera *Rhinocladiella* and *Exophiala* represented genetically identical, but morphologically different anamorphs of one species. One sequence type (#145) was a possible candidate for the genus *Cladosporium* found by culture, yet its ITS-1 area had a higher (99%) similarity with a *Coniothyrium sp.* isolate. Some of the species identified only by sequencing may have been among the unidentifiable morphotypes on culture plates (e.g. basidiomycetous *Bensingtonia* that has yeast-like cell form). By the described RFLP classification and random sequencing of one representative from each class 74% of the different ITS types would have been identified. Evaluation of the effectiveness of this method is hindered by the presence of chimeras, which are not natural DNA molecules. This study was done as a preliminary assay to find a suitable method to screen for fungal diversity in building material samples. The protocol was successful in finding uncultivable species in the sample, but some limitations still affect the routine use of molecular species identification. The main factors concern methodology-based errors, costs of handling large DNA libraries and limited size of the public DNA databases. Additionally, traditional PCR does not produce quantitative results (von Wintzingerode *et al.* 1997). In this study, the most probable source of PCR artifacts was in the PCR conditions, which were later optimized to remove the problem. Low cycle number, long elongation time, high concentration of primers and nucleotides and use of proofreading DNA polymerase in primary PCR produced libraries free of chimeras (unpublished data). Screening for different inserts is a prerequisite for handling large libraries at sensible costs. Due to extensive variation in sequence and length of the ITS area, simple electrophoretic separation or RFLP-based application has in many cases been considered efficient to separate between different targets (Chen *et al.* 2000; Hendolin *et al.* 2000).

The following conclusions were a result of the study:

- 1) Species unattainable by cultivation can be detected using the method described. However, the number of the clones screened was most probably not large enough to cover all the species present in the sample. The two methods complement each other in species identification.

- 2) The PCR conditions and the heterogenic template material used here cause the formation of remarkable amounts of chimeric PCR products, which create false genetic diversity to the material. The origin of the different parts of the products can nevertheless usually be determined. The PCR-conditions must be optimal to reduce the frequency of chimeras.
- 3) The possibility of identifying and naming organisms on the basis of their DNA sequences is, to a great extent, dependent on the size, coverage and reliability of the existing sequence database to which the analyzed sequences are compared. In the future, as the databases get larger, the methods used here become more specific and efficient.

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FUNGAL ENZYMES IN INDOOR DUST

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ABSTRACT

Concentrations of the enzymes NAGase and β -glucosidase in airborne dust, exhaust duct dust, and floor dust from 15 schools were studied. NAGase was detected in the 352 studied dust samples and β -glucosidase in 23 of 50 studied samples. NAGase activity/g dust was higher in airborne (gm=12823 pmol/sek/g) than in floor (gm=13 pmol/sek/g) and duct (gm=18 pmol/sek/g) dust. Higher NAGase activity per floor area was found in rooms with than without carpets. NAGase activity correlated significantly with cfu of fungi ($r=0.81$, $n=352$). The amount of released NAGase from *Penicillium chrysogenum* colonised gypsum boards as affected by air exposure increased with increasing velocity of air and correlated with numbers of released conidia. Whether NAGase in the found concentrations can cause health effects is not known, but the findings lead to a suggestion of studies of whether the NAGase assay provides a useful epidemiological tool for investigating associations between fungal exposure and health.

INDEX TERMS: cfu of fungi, chitinase, dampness in buildings, quantification of fungi

INTRODUCTION

Several studies have indicated an association between the occupancy in fungal contaminated buildings and various health symptoms (Pasanen *et al.* 2001). However, a review study concluded that the role of fungi in health effects associated with dampness in buildings is not clear (Bornechag *et al.* 2001). This finding may be due to lack of valid methods to assess fungal exposure. Valid methods for measuring airborne fungi are crucial to research and hazard identification. Airborne microorganisms are often quantified by counting using microscopy and/or cultivation (Douwes *et al.* 1999; Macher, 2001). However, these methods can underestimate the actual number due to aggregation or inadequate staining of spores (Eduard, Aalen, 1988; Heldal *et al.* 1996) and the fact that not all fungi are cultivable or iden-

tifiable as being fungi. The general markers for fungal biomass, ergosterol, $\beta(1\rightarrow3)$ -glucans, and polysaccharides of *Aspergillus spp.* and *Penicillium spp.* (ESP-Asp/Pen), have also been applied in studies of exposure to fungi (Douwes *et al.* 1999; Miller, 1997; Rylander, 1996; Szponar, Larsson, 2001), but these methods have not been well validated Douwes *et al.* (2003).

Instead of quantifying fungi or markers of fungi, exposure to particular toxic or allergenic constituents and metabolites may be determined. One approach could be to quantify fungal enzymes as several fungal enzymes are known to have caused respiratory effects (Jeffrey *et al.* 1999; Kauffman *et al.* 2000; Losada *et al.* 1986; Robinson *et al.* 1990) and as the concentrations at which fungal enzymes are present in indoor dust are not known. Recently, Madsen (2003) found a significant correlation between total number of fungi in airborne dust and NAGase (N-acetyl- β -D-glucosidase) activity ($r=0.802$, $n=76$, $p<0.0001$). In the same study NAGase activity also correlated significantly with the numbers of culturable fungi. Thus, it is hypothesised that NAGase activity may be a marker of fungal spores and other fungal components in dust. In addition to the found correlation the arguments are: 1) that common indoor fungi as *Aspergillus*, *Alternaria*, *Penicillium* and *Trichoderma* secrete the enzyme NAGase (Clayessens, Ayerts, 1992; Draborg *et al.* 1995; Hearn *et al.* 1998; Lahoz *et al.* 1976); 2) NAGase is expected to be secreted by all chitin containing fungi (Gooday *et al.* 1992), 3) NAGase is stable in dust at room temperature (Madsen, 2003). Furthermore the method is cost effective and NAGase activity can be expressed in a SI unit.

In this study, NAGase is quantified in airborne, floor and exhaust duct dust sampled in 15 schools (the dust origins from the Danish multi centre study named Molds in Buildings) and in *Penicillium chrysogenum* aerosols in order to obtain information of the levels of NAGase in indoor dust. Of the 406 school dust samples analyzed for NAGase content 50 randomly chosen samples were investigated for content of β -glucosidase. Correlations between the contents of NAGase and fungi are examined.

METHODS

Collection of school dust: Airborne, floor and ventilation duct dust were collected on 15 Danish schools in the winter 1999 to 2000 (Meyer *et al.* 2004). Dust was collected in 5-8 rooms at each school. Samples were analyzed for NAGase activity and culturable fungi and randomly selected samples also for β -glucosidase activity.

Airborne dust: bioaerosols were sampled by stationary sampling for approx. 5 hours using IOM inhalable dust samplers (airflow of 2 L/min) mounted with polycarbonate filters (25 mm; 0.4 μ m; Nuclepore). Samplers were placed 1.5 m above the floor.

Floor dust: dust from the floors was collected using a modified HVS-3 sampler (ASTM, D5438-94) connected to a vacuum cleaner. The dust was separated from the air by a cyclone. Floors were vacuumed without moving the furniture. A bicycle computer (cyclocomputer, Cateye vectra, model cc-7000) measured the vacuumed distance. The floors were mopped every day in the morning or late afternoon.

Exhaust duct dust: dust from the ducts was sampled using a VacuMark-sampler mounted with paper filter or the dust was carefully brushed down in foil boxes.

Extraction of dust: Filter cassette samples were kept at room temperature for a maximum of 24 hours and then resuspended in 10 ml sterile 0.05% Tween 80. The cassettes were shaken for 15 min (500rpm). Floor dust samples were suspended in 0.05% Tween 20 with shaking (250 rpm) for one hour. The suspensions were used immediately for cultivation and the remainder of the samples was frozen at -80°C for enzyme assays.

Enzyme assays: To quantify the activities of NAGase (EC3.2.1.30) and β -glucosidase (EC 3.2.1.21) release of *p*-nitrophenol from *p*-nitrophenol-N-acetyl- β -D-glucosaminide or *p*-nitro-phenol-N-acetyl- β -D-glucopyranoside (Sigma Chemical Co. U.S.A.) was quantified according to Madsen and Neergaard (1999). Appropriate controls without either the enzyme or the substrate were run simultaneously. One unit of enzyme activity is defined as the amount of enzyme, which releases 1 mmol of *p*-nitro-phenol ml^{-1} enzyme min^{-1} . Activities are expressed as pmol sek^{-1} per g dust or per m^3 air or per m^2 floor.

Culturable fungi: Dilutions of the dust suspension were plated onto Dichloran Glycerol Agar (Oxoid CM729) with penicillin (30mg/L) and streptomycin (30 mg/L). The number of colony forming fungal units (cfu) was calculated and reported as cfu/ m^3 air, cfu/g dust and cfu/ m^2 floor as applicable for the sample.

Aerosolization of *Penicillium chrysogenum* as affected by different air velocities: *Penicillium chrysogenum* (Thom) was grown on 0.08 m^2 of sterilized wet wallpapered gypsum boards. Two hundred milliliters distilled milliQ water (Millipore, Glostrup, Denmark) was used for wetting the boards. Inoculation was performed by spraying 1 ml conidium suspension (10^6 conidia/ml) with 1 g peptone per 100 ml milliQ water onto the piece of gypsum board using an atomizer. Following inoculation, the gypsum boards were placed in stainless steel boxes with tightly fitting glass covers. The fungus was incubated at a RH of approximately 95% for 34 days at 20-22 $^{\circ}\text{C}$. A saturated solution of potassium sulfate controlled the RH. A P-FLEC (particle-field and laboratory emission cell) (Chematec, Denmark) was used for measuring the release or re-suspension of particles from surfaces at an adjustable airflow. The release of conidia and NAGase was measured as a function

of airflow over the surface (0.3; 0.5; 1.0; 1.5; 2.0 and 3.0 m/s) to see whether there was an association between numbers of released conidia and NAGase activity. Aerosols were sampled on 0.4mm polycarbonate filters. The dust was extracted and NAGase quantified and the total numbers of conidia were quantified using a haemocytometer.

Statistical analysis

Statistical analyses were performed with SAS (version 8e, SAS Institute, Cary, NC). NAGase and cfu data were normal logarithm transformed. Mean concentrations were expressed as geometric means (GM) and standard deviations as s*. Pearson correlations (r) were calculated between cfu or total number of fungi and NAGase activity and between the average relative humidity and airborne NAGase. Statistical testing comparing NAGase levels was performed with a *t* test on normal logarithm-transformed data.

RESULTS

NAGase was present in the dust samples, in the air and on the floor in very different amounts (Table 1, Fig. 1). β -glucosidase was found in 23 of 50 samples with the highest concentration in airborne dust (173 pmol/sec/g). The standard deviation s* of airborne NAGase/m³ from all schools was 2.33 and inside each school it was between 1.23 and 2.22. The standard deviation s* of NAGase/m² floor of all schools was 2.57 and inside each school it was between 1.15 and 2.25.

Table 1. NAGase activity (pmol/sec) in school dust.

Dust	N	NAGase/g dust			NAGase/ m ² floor			NAGase/ m ³ air		
		GM	Interval	s*	GM	Interval	s*	GM	Interval	s*
Floor	119	13	1-889	3.04	1.0	0.3-5.7	2.56	-	-	-
Air	182	12823	255-1x10 ⁵	2.73	-	-	-	0.88	0.12-6.3	2.33
Duct	51	18	3-551	2.93	-	-	-	-	-	-

NAGase activity/g dust was higher in airborne dust than in floor and duct dust (Table 1). NAGase was measured in dust samples from staff rooms (GM=1.5 pmol/sek/m², n=17), and 8 of the samples were from rooms with (GM=2.8 pmol/sek/m²) and 9 from rooms without (GM=0.82 pmol/sek/m²) carpets. Significantly higher NAGase activity was found per floor area in rooms with than in rooms without carpets (p=0.0317). Furthermore, the cfu per NAGase unit tended to be higher in staff rooms with (gm=2997cfu/(pmol/sek)) than without (gm=849 cfu/(pmol/sek)) carpets (p=0.06).

Table 2. Correlation between NAGase activity and cfu of fungi in school dust and between NAGase activity and total number of *P. chrysogenum* conidia.

NAGase in	n	cfu/g dust	Cfu/ m ² floor	Cfu/ m ³ air	<i>P. chrysogenum</i> conidia
Floor dust*	119	r=0.34, p=0.0002	r=0.29, p=0.0016		
Airborne dust*	182	r=0.40, p<0.0001		r=0.34, p<0.0001	
Dust from ducts*	51	r=0.36, p=0.0095			
Total school dust*	352	r=0.81, p<0.0001			
Aersols from <i>P. chrysogenum</i> cultivated gypsum boards	12				r=0.89 p=0.0001

*Floor, air and duct dust from the 15 schools.

Significant correlations were found between NAGase activities and cfu of fungi (Table 2). The cfu per NAGase unit was higher in dust from floors and ducts than in airborne dust ($p<0.05$). The exposure of *P. chrysogenum* growing on gypsum boards to different air velocities affected the amount of aerosolised NAGase and conidia (Fig. 2), and NAGase activity correlated significantly with the total number of *P. chrysogenum* conidia (Table 2).

Figure 1. Relative humidity versus airborne NAGase activity (gm) (pmol/sek/m³) ($r=0.62$, $p=0.014$). The signature letters are codes of each school, and the following number the average air temperature.

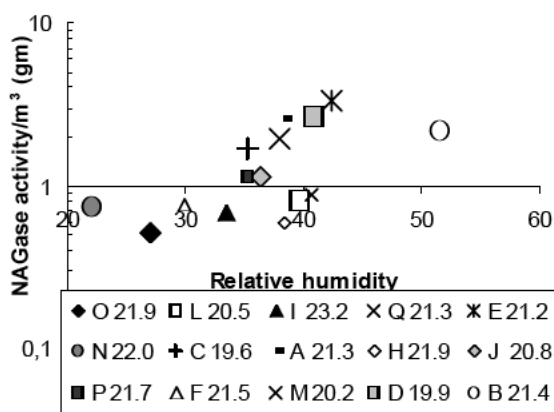
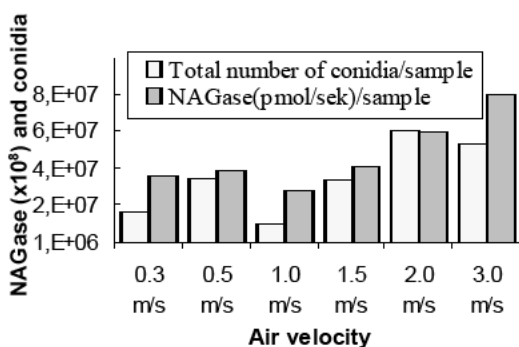


Figure 2. Total number of aerosolised *P. chrysogenum* conidia and NAGase as a function of exposure to different air velocities. Data of numbers of conidia are from Kildesø *et al.* (2003).



DISCUSSION

Different studies have argued for using either measurements of microorganisms in the air, floor or duct dust in studies of exposure (Marcher, 2001), and recently a method (P-FLEC) which may be used for measuring release of fungal spores of moldy surfaces has been introduced (Kildesø *et al.* 2003). Therefore, NAGase, was quantified in this study in these four kinds of dust and compared to culturable data. Exposures to NAGase in schools were between 0.12 and 6.3 pmol/sek/m³. The only other study reporting exposure to NAGase was performed at a biofuel

plant and the exposure at the plant was between 3 and 56 pmol/sek/m³ (Madsen, 2003). At the office at the biofuel plant the median exposure was 3.6 pmol/sek/m³ (Madsen, 2003) and this exposure to NAGase is 4 times higher than the exposure (GM) found in this study at schools. NAGase per g airborne school dust was at the level Madsen (2003) found in biomass dust (200-5x10⁵ pmol/sek/g dust), while the content of NAGase in school floor and duct dust were lower than in biomass dust. Whether the levels of NAGase and β -glucosidase found in this study are involved in the health effects that fungi can cause in indoor environments is not known, but studies in environments where materials containing fungal enzymes are handled show that different fungal enzymes can cause respiratory disorders and allergy (e.g. Blanco *et al.* 1999; Doekes *et al.* 1999; Houba *et al.* 1996; Jeffrey *et al.* 1999; Kauffman *et al.* 2000; Losada *et al.* 1986; Posch *et al.* 1999; Robinson *et al.* 1990).

NAGase activity was significantly correlated with of cfu of fungi and in the P-FLEC experiment with total numbers of *P. chrysogenum* conidia. The correlation between cfu and NAGase activity in dust from ducts, floor and air was at the levels with correlations between cfu and EPS-Asp/Pen in house dust found by Douwes *et al.* (1999). However, when data from floor, duct and air were pooled (total school dust) a higher correlation was found than in the study with ESP-Asp/Pen. The correlations between NAGase activity and cfu of fungi in air, floor or duct dust samples were lower than the correlations found by Madsen (2003) between cfu and NAGase activity in biomass dust. However, the correlation between total school dust and cfu was higher than the correlations reported in Madsen (2003). The high correlation between total numbers of *P. chrysogenum* conidia and NAGase activity is in accordance with studies showing that NAGase correlates stronger with total number of fungi than with cfu of fungi (Madsen, 2003). The airborne NAGase activity correlated also with the relative humidity of each school. Similarly, Douwes *et al.* (1999) found a significant correlation ($r=0.54$) between EPS-Asp/Pen in mattress and humidity in bedrooms of 60 homes.

The NAGase activity/g dust was significantly higher in airborne than in floor and duct dust. The lower NAGase activity per g floor and duct dust than per g airborne dust, may be caused by presence of larger particles in the floor dust which were absent in airborne dust together with the observation of Marcher (2001), that fungi are more abundant in the fine than the fibrous fraction of floor dust. The very different concentrations of NAGase in floor and airborne dust (Table 1) show different composition of airborne and floor dust and only a small fraction of the floor dust may have been re-suspended. Similarly, Kildesø *et al.* (1999) suggested that particles tracked into buildings on shoes tend to be large and are unlikely to be re-suspended once deposited.

The cfu per NAGase unit was higher in dust from floors and ducts than in airborne dust. This may reflect a greater cultivability of fungi from duct and floor dust than from airborne dust. The apparently lower cultivability of airborne fungi may be caused by 1) the stress aerosolisation causes, 2) flow on the filter during 5 hours of sampling of airborne dust, 3) particles in floor and duct dust are larger than in airborne dust and the spores may be more protected (from e.g. UV-irradiation and drying) in larger particles.

Carpets may be protective environments for fungi compared to floors without carpets. This was reflected in the tendency to a higher cfu per NAGase unit in staff rooms with than without carpets. Furthermore, significantly higher NAGase activity per floor area was found in staff rooms with than areas without floor carpets. Carpets are also in other studies identified as important dust reservoirs (Leese *et al.* 1997; Kildesø *et al.* 1999) and different studies have reported higher amounts of fungal components per g dust from textile floor coverings than from smooth floors (Douwes *et al.* 1999; Verhoeff *et al.* 1994).

CONCLUSION AND IMPLICATIONS

The enzyme NAGase was found in the 406 dust samples from 15 schools showing that people are exposed to this enzyme in indoor environments (0.12-6.3 pmol/sek/m³ air). Whether NAGase and β -glucosidase in the found concentrations cause any health effect is not known, but studies of pulmonary health effect of NAGase are in progress. The facts that NAGase activity: 1) is associated with spores of indoor air fungi, 2) could easily be quantified in indoor dust and the method is cost effective, 3) correlated with number of fungi, 4) is associated with both cultivable and non-cultivable fungal components and 5) is stable in dust at room temperature, leads to a suggestion of further studies and documentation of whether the NAGase assay provides a useful epidemiological tool for investigating the relationship between dampness in buildings, buildings with high fungal contamination, fungal exposure and health effects.

ACKNOWLEDGEMENTS

The Danish Research agency and the Danish Working Environment Council (ASC) are acknowledged for financial support, and M. Simkus, A. Pourzenal and J. H. Peitersen for their help.

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RAPID DETECTION AND QUANTITATION OF FUNGAL SPORES FROM DUST SAMPLES USING REAL-TIME PCR

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ABSTRACT

Recent advances in real-time PCR have permitted accurate, rapid and quantitative identification of microorganisms in pure cultures regardless of viability or culturability. In this study, a simple sample processing method was investigated for rapid identification and quantitation of fungal spores from dust samples using real-time PCR. The proposed method was evaluated for susceptibility to interference from environmental dust samples. The extent of inhibition was calculated using real-time PCR reactions containing *Aspergillus fumigatus* spores specific primers and probe and various amounts of dust. No interference ($p < 0.05$) was detected from 0.2 mg of four real-world dust samples. However, dusts weighing > 0.2 mg compromised the assay. The overall results suggest the potential usefulness of our method for monitoring indoor microbial aerosols containing dusts weighing ≤ 0.2 mg using real-time PCR.

INDEX TERMS: Quantitative PCR, Fungal spores, Dust samples, TaqMan

INTRODUCTION

Fungal aerosols are found in many types of indoor environments including homes, hospitals, and offices. They have been implicated in detrimental health effects. Therefore, accurate estimation of exposure to airborne fungi is important. Traditional detection methods have mainly relied on microscopic and culture-based techniques, both of which have limitations. Microscopy requires technical expertise and is highly subjective. Culture-based methods are biased towards identification of rapidly growing fungi and fungi in high concentrations, and there is no ideal medium for growth of all fungi. In addition, non-culturable/non-viable spores are not detected by culture, but can be allergenic causing health problems. Thus, there is a need for better techniques to identify environmental exposures to fungi.

Recent developments in molecular techniques such as nucleic acid amplification methods including polymerase chain reaction (PCR) have provided significant advances in rapid detection and characterization of microorganisms irrespective of their viability or culturability. PCR has been demonstrated to be robust, flexible, sensitive and accurate for detection of over a hundred of the most common indoor fungi (Kahane *et al.* 2002). The majority of studies on real-time PCR have been performed using pure cultures grown in the laboratory and have not considered the effect of contaminants that might be present in environmental samples. A few studies have focused on removal of inhibitory compounds from samples using commercially available nucleic acid extraction kits (Haugland *et al.* 2002; Cruz-Perez *et al.* 2001a, b). Each additional step adds time and complexity, and generally reduces the DNA yield. Some investigators have added enhancement agents to overcome PCR inhibition (Bickley, Hopkins, 1999) and others have simply used serial dilution (Fode-Vaughan *et al.* 2001).

Previous studies in our laboratory investigated a simple sample processing method using 20% nutrient broth to maintain the specificity and sensitivity of conventional PCR and to overcome PCR inhibition in various dusts collected from a poultry house, hospital rooms, and HVAC filters. Those results showed no interference from dusts weighing 0.2 mg with a 10-fold dilution. The focus of the present research was to expand that study to real-time PCR. The dusts were sieved to represent the aerosolizable fraction and were spiked with spores of *Aspergillus fumigatus*. This fungus was chosen as a test organism due to its adverse health effects in indoor environments, and availability of species specific primers and probe for real-time PCR (Haugland *et al.* 2002). Detection sensitivity and reproducibility of the method was evaluated.

METHODS

A total of four dusts were tested for interference; settled dust collected from inside a heater in a poultry house, settled dust on chairs from two hospital rooms, and dust on filters from a commercial HVAC system (roof-mounted filters). The dusts were sieved through a 425- μm mesh (Sieve Shaker, W. S. Tyler Inc., Mentor, OH) to represent the aerosolizable fraction of dust. The dust amount ranged from 0.2 to 2 mg/sample.

Aspergillus fumigatus 17-30-37 was provided by Janet Simpson (NIOSH, Morgantown, WV) and was cultured on Malt Extract Agar (MEA, Difco 325, Difco laboratories, Detroit, MI, U.S.A.) plates at 24°C at a relative humidity of 32-40% for 10 days. The fungal spores were collected by gentle rolling of moistened, sterile cotton swabs over the surface of the colonies. Amounts ranging from 2×10^3 to 2×10^5 spores/sample were added directly to a sterile Eppendorf tube con-

taining 20% nutrient broth, known amounts of dusts and zirconia/silica beads. After sample processing, 200 spores corresponded to 1 spore DNA equivalent/PCR reaction.

To compare PCR amplification from crude extract and purified DNA of *A. fumigatus* spores, both preparations were made from the same stock of spores. The crude extract of spores with and without dust was prepared by the general method of bead-beating as described by Zhou *et al.* (2000) with slight modification. The modifications included addition of 0.3 g of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK, U.S.A.) to 2.0 ml screw-cap tubes, 0.6 ml of 20% nutrient buffer, 0.1 ml of spore suspension of known concentration, and known amounts of dust. Standard curve was prepared using crude extract from 2×10^2 to 2×10^6 spores/ml in sample. Four independent reactions were performed to determine the correlation between cycle number and initial copy number of templates [log (spore DNA equivalents)].

DNA was purified by FastDNA kit (Bio 101, Vista, CA) using the manufacturer's protocol with slight modifications to correspond to the protocol of crude extract preparation. The lysing matrix from the kit was substituted by the same amount and type of beads as mentioned before and added to similar 2.0 ml screw-cap tubes. The volume of CLS-Y solution was reduced to 0.6 ml from 1.0 ml. The samples were homogenized in a Mini-Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.). The remaining steps were followed as mentioned in the manufacturer's protocol with respect to DNA purification using the spin filters and catch tubes in the kit.

The TaqMan assay was prepared and performed using previously designed primers and probes for *A. fumigatus* as described by Haugland *et al.* (2002). PCR inhibition was calculated by comparing the threshold cycle (or Ct value which indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold) obtained from samples with and without dust to an external standard curve linear over a 5 log scale. Each experiment was replicated twice, with PCR reaction from each sample being performed in duplicates.

Statistical methods were used to evaluate reproducibility of the experiments. The data analysis for this study was performed using SAS/STAT software, Version 8.2 of the SAS System for Windows (SAS Institute, Cary, NC). The standard curve regression equation was generated with Proc Reg utilizing the Ct values and natural log of the known concentration of spores. Differences between treatment groups were analyzed using a 3 (Spore concentration) by 5 (dust concentration) mixed model analysis of variance (Proc Mixed). Differences were considered significant at $p < 0.05$. To determine when the concentration of dust begins to inhibit

it the recovery of spores from the reaction, 95% confidence intervals were calculated.

RESULTS

The sensitivity of previously developed primer and probe set to detect *A. fumigatus* DNA in a crude DNA preparation was evaluated by two independent real-time PCR experiments. In one experiment, crude DNA extract was diluted ten fold serially. In the other experiment, crude DNA extract was directly obtained without dilution from specific number of spores. Each experiment was replicated twice, with PCR reaction from each dilution being performed in duplicates.

Figure 1(A). Comparison of Ct versus Log (Spore) for purified and crude DNA

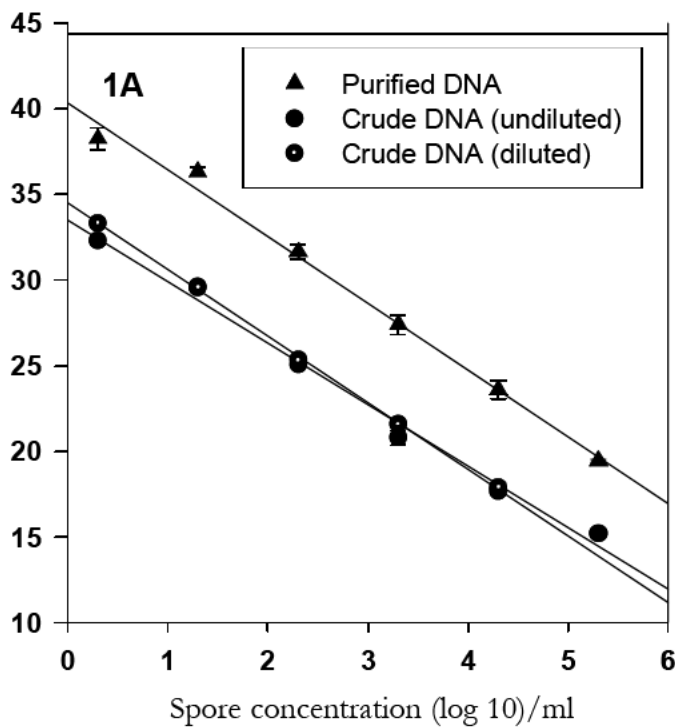


Figure 1(B) Comparison of Ct versus Log (Spore) for purified and crude DNA

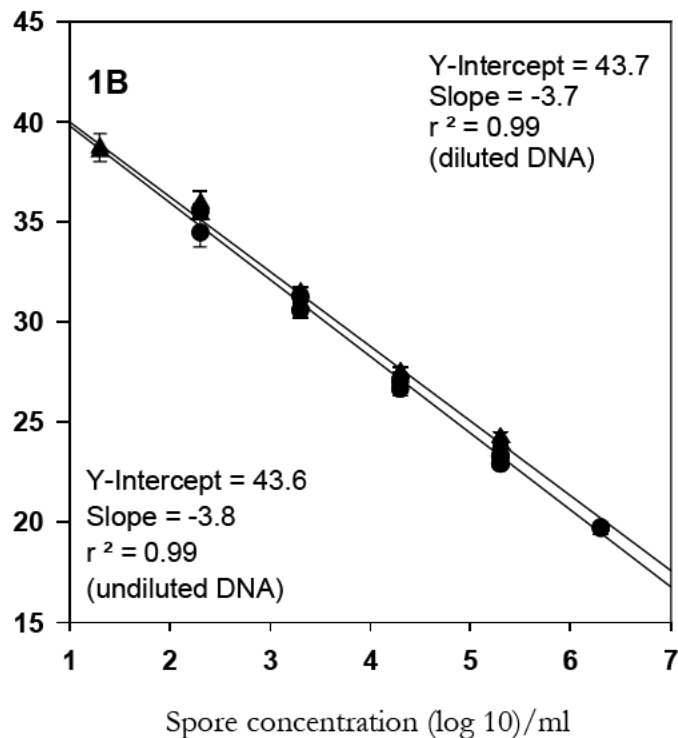


Figure 1A demonstrates that there is a correlation between target DNA concentration and changes in fluorescence over six orders of magnitude ($y = 34.5 - 3.8x$, $r^2 = 0.999$ for serially diluted crude DNA; $y = 33.5 - 3.6x$, $r^2 = 0.991$ for undiluted crude DNA). The standard curve from purified DNA was also linear over six orders of magnitude and parallel to that of crude DNA. However, the Ct values at each spore concentration were higher, indicating lower sensitivity ($y = 43.6 - 3.8x$, $r^2 = 0.99$ for undiluted DNA).

For comparison of real-time PCR in the presence or absence of dust, another standard curve was obtained using crude DNA from spores ranging from 2×10^2 to 2×10^6 spores/ml. These concentrations were chosen to encompass the range of spores normally encountered in indoor air. The results in Figure 1B show linearity over 5 orders of magnitude and reproducibility of results with replicate data points very close together. A 10-fold dilution of the samples mentioned above also resulted in a linear standard curve, with a sensitivity of detection of 0.1 spore DNA equivalent/PCR reaction.

A detailed study using real-world samples collected from a poultry farm was performed to investigate interference on real-time PCR. Dust samples ranging in

weight from 0.2 to 2.0 mg were spiked with 2×10^3 to 2×10^5 *A. fumigatus* spores/ml in sample.

Figure 2. *A. fumigatus* spore concentration in dust based on standard curve from real-time PCR.

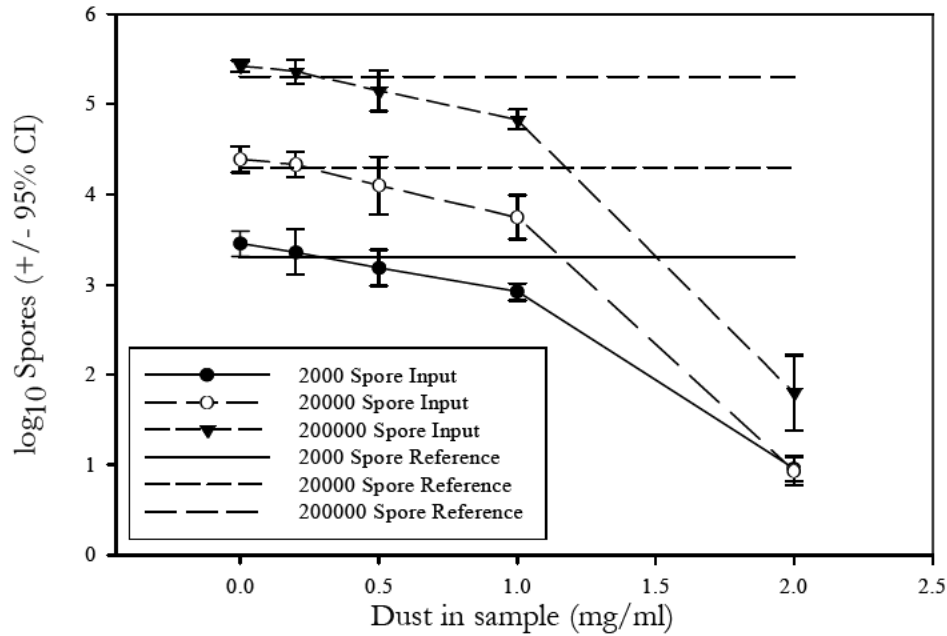
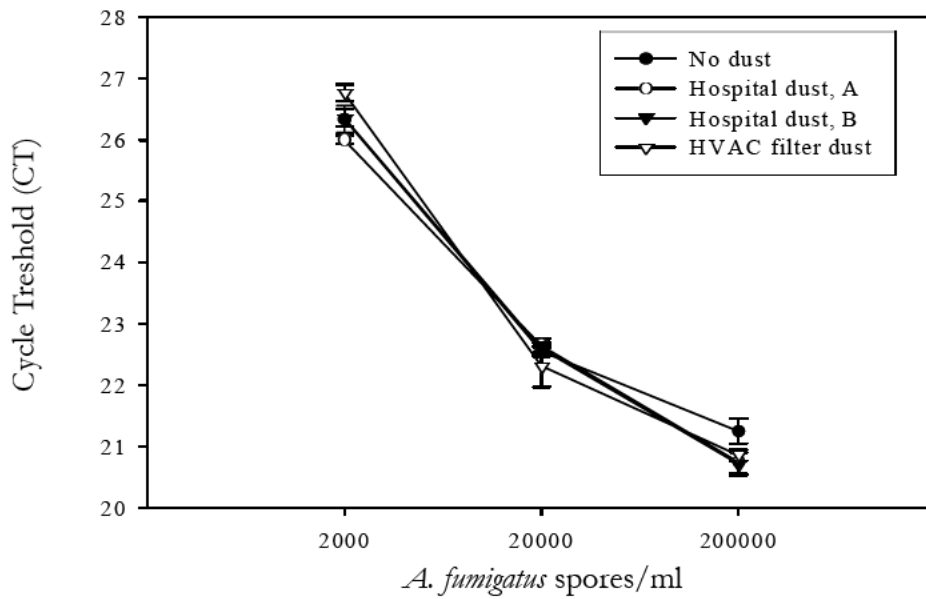


Figure 2 shows that the extent of inhibition by poultry dust on real-time PCR varies with amount of dust. For 0.2 mg of tested dust, no statistically significant differences were detected with undiluted or 10-fold diluted samples ($P > 0.05$). For 0.5, 1 and 2 mg dusts, reduction in spore number (inhibition) compared with values determined in samples without dust was statistically significant ($P < 0.05$). However, the number of spores recovered from 0.5 mg dust was within 95% confidence intervals.

A preliminary study using 0.2 mg of 3 additional real-world dust samples collected from two hospital rooms and commercial HVAC filters were studied to evaluate the pattern of inhibition. No interference was detected from the 3 additional dusts with undiluted samples at p -value > 0.05 (Figure 3).

Figure 3 Comparison of Ct versus Spore concentration for 0.2 mg of 3 dusts.



DISCUSSION

A simple sample processing method for real-time PCR assay has been developed and evaluated in our laboratory. It has the potential for monitoring microbial aerosol in indoor environments in conjunction with rapid, sensitive and specific real-time PCR. The method includes bead-beating in the presence of nutrient broth, six times in excess of spore volume, a PCR enhancer, and a primer-probe set for real-time PCR.

The presence of contaminants in environmental samples can interfere with real-time PCR assay to varying degrees leading to false negatives or underestimation of microbial load. Most of the studies employing real-time PCR reported recently in the literature focused on purification of DNA from the samples (Haugland *et al.* 2002; Cruz-Perez *et al.* 2001a, b). DNA purification methods affect the detection limits of PCR-based methods either due to inefficient DNA extraction or loss of DNA during the process as seen by the variability in results (Poussier. *et al.* 2002). Also, despite elaborate purification steps, sometimes it is necessary to dilute the DNA preparations (Cruz-Perez *et al.* 2001a, b).

In this study, we used crude DNA extracted in the presence of nutrient broth and bovine serum albumin (BSA) to maintain the integrity of samples with minimal manipulations. Nutrient broth, six times in excess of spore volume served as a dilu-

ent to overcome inhibition. Proteins from beef extract and peptone in the nutrient broth and BSA presumably act as scavengers for inhibitory compounds, preventing them from binding to and inactivating Taq DNA polymerase. Both, peptone and BSA have been reported earlier as enhancers of PCR (Bickley, Hopkins, 1999). These proteins may also nonspecifically increase PCR efficiency by preventing released fungal DNA from sticking to the beads used to lyse spores. Our results showed no interference using 0.2 mg of four different dusts.

For bioaerosols collected from environments with dust concentrations of $< 100 \mu\text{g}/\text{m}^3$ (typical of indoor environments) using a sampling flow rate of 2 l/min for 6 hour sampling time less than 0.072 mg of total dust is collected. Because 0.2 mg of various dusts did not show any inhibition, our method has the potential to monitor bioaerosols from indoor environments collected using low-volume samplers over a period of hours. The number of target organisms evaluated in dust samples was based on calculations using average fungal spore level in indoor environments as described by Zhou *et al.* (2000). The sensitivity of detection was as low as 200 spores/sample corresponding to 1 spore DNA equivalent/PCR reaction using a 10 fold dilution.

CONCLUSIONS AND IMPLICATIONS

The method proposed in this study has a sensitivity of detecting low levels of spores with sample processing achievable within one hour, followed by real-time PCR performed in three hours which includes set up of PCR-reactions. Our study used four different types of dusts spiked with *A. fumigatus* spores. This technique was tested using duplicate and replicate samples and shown to be highly reproducible. The sample processing method can be used in conjunction with any previously designed species specific primers and probe sets for real-time PCR. Thus the work can likely be extended to all the fungi in indoor environments that have been implicated in adverse health effects as long as the dust sample size is ≤ 0.2 mg.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Drs. M. Harper, D. Lewis and D. Weissman at NIOSH for their advice and review of this paper. Poultry dust was provided by Steven Lenhart (Cincinnati); Hospital rooms and HVAC dusts were provided by Carol Rao (NIOSH, Morgantown, WV).

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Chapter 9
Transitional Countries

Session chairs:
Nceba Gqaleni, Leila Brickus

THE INDOOR ENVIRONMENT AND ALLERGY AS POTENTIAL STRESSORS IN DURBAN HOSPITALS, SOUTH AFRICA

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ABSTRACT

This study reports on six Durban hospitals establishing indoor environmental conditions, allergy, and stress among 280 staff. The presence of bioaerosols, surface molds, allergy stressors, potential for discomfort from levels of carbon dioxide, temperature, relative humidity and air movement were determined. In each hospital certain areas had a potential of exceeding the 1000ppm CO₂ level, suggesting that ventilation systems were not performing adequately. All the hospitals had sources of fungal growth. Many of the ceilings and walls in these public hospitals had areas greater than 3m² contaminated with fungi. Most predominant organisms included *Aspergillus*, *Cladosporium*, *Penicillium*, and other fungal species. More than 50% of staff were stressed while 32.5% of the staff reported that they experienced allergies.

INDEX TERMS: Hospitals, molds, allergy, stress, indoor environment

INTRODUCTION

Poor indoor air quality may have a psychological association (Burton, 1991). Stress in nursing staff in Valencia, Spain lowers their psychological welfare (Escribà *et al.* 2000). Stress makes the immune system less efficient and failure to cope with stress causes poor health and negative performance (Schlebusch 2000). Individual responses within a hospital environment may vary and suggest that bacteria and fungi that can spread within the air supply systems may reduce immune system resistance (Gill, Wozniak, 1993). Common allergy provoking molds in South Africa include *Cladosporium*, *Aspergillus* and *Penicillium* (Bryant 2001). House dust mites

tend to cause the most allergies in South Africa. No known research had been conducted in South African hospitals on the occurrence and impact of molds. Preliminary investigations assisted in establishing the indoor air variables to be studied in hospital environments (Shadwell *et al.* 2000). Consideration was given to the performance of ventilation systems and building maintenance programs. The objective of this paper is to discuss the descriptive results of the following hospital staff stressors:

- Fungal contamination and allergy
- Indoor air quality measurements
- Staff comfort, general hospital hygiene and maintenance concerns
- Psychological stress

METHODS

Durban has 23 hospitals of which 30% (7) are public, 13% (3) are semi-private and the remaining 13 (57%) are private. Approaches were made to all Durban hospitals (N=23) in close proximity to each other so that climatic conditions would be similar and psychosocial issues would relate to the Durban residential areas. Six (n=6) hospitals (30%) were randomly chosen and stratified to represent, two public hospitals (public 1 and public 2), two semi-private hospitals (semi-private 1 and semi-private 2) and two private hospitals (private 1 and private 2). Work areas examined were: administration, Intensive Care Units (ICU), three general wards, laundries, pharmacies, Central Sterilising and Supply Departments (CSSD), kitchens and theatres. All willing staff in the selected areas were included (N = 280 being 87% of the total staff working in the sections surveyed). Acceptable comfort standards were: temperature 22.5 to 26°C; air movement 0.1 to 0.3m/s; humidity 40 to 60%; CO₂ below 1000ppm (ASHRAE Standard No 62-1989, ASHRAE Standard No 55-1992 and London Hazard Centre, 1990). Surface fungi and fungal bioaerosols were sampled as previously published (Gqaleni *et al.* 1999). Real-time particulate measurements levels were used as an indicator of cleanliness. The monitor recorded particles per litre measuring particle sizes in microns as recommended (International Standard ISO 14644-1). A Kata thermometer, wet bulb thermometer, stop watch and distilled water heated to 50°C were used to measure air velocity (Shadwell *et al.* 2000).

Skin prick allergy tests established allergy potential to common fungi, dust mite and the German cockroach. Allergen testers were based on previous allergy research by (Gqaleni *et al.* 1999). An allergy questionnaire was based on research by (Randolph, 1998), (Gqaleni *et al.* 1999), (Baraniuk *et al.* 1998) and (Weinberg *et al.* 2002). A psychological questionnaire, the Stress Symptom Checklist (SSCL),

designed and previously published by (Schlebusch 2000) assessed biopsychosocial stress. The SSCL was tested for reliability and validity using the Tension and Effort Stress Inventory (TESI) (Svebak, 1993), the Psychosomatic Ailments Questionnaire (PSYSOM) (Hinton, 1991) and a ten point likert stress scale. A "Hospital Environmental and Personal Stress Assessment Questionnaire" established physical indoor stressors, smoking habits, frequency of colds and influenza, the occurrence of allergy, lack of control over the work environment and other physical ailments and was developed from questionnaires designed by (Raw, 1995) and (Driscoll *et al.*1992).

RESULTS AND DISCUSSION

Fungal surface contamination: Predominant viable surface fungi were: *Aspergillus flavus*, *A. ustus*, *A. niger*, *A. syndowii*, *A. trichoderma*, *A. ochraceus*, *A. versicolor*, *A. japonicus*; various *Penicillium* species with the specific identification of *P. citrinum* and *P. chrysogenum*; *Fusarium* species; *Cladosporium herbarium*; *Rhizopus*, *Moniliella*; *Geotrichum candidum* and *Mucor*. These were found on: ceilings, fans, air condition outlet and inlet surfaces, shelves, solid floors, carpets, wooden windows and wood chip in plant boxes. *Aspergillus spp* and *Penicillium spp* were ubiquitous in the same sections on contaminated surfaces and in air samples. Both public hospitals had more sections where fungal contaminated surfaces were greater than 3m² compared to the semi-private and private hospitals. Two public hospital both had eight sections contaminated with fungi with soiled surface areas ranging from .2 to >3m². In semi-private hospital 1 there were seven contaminated surface areas ranging from .2 to >3m². Semi-private hospital 2 and private hospital 1 had only one area with a fungal surface area greater than 3m². In public hospitals, it was evident from observations and comments from staff that maintenance programs were inadequate. Flanningan and Morey (1996) suggest that fungal contaminated surface areas >3m² must be fully contained and negatively pressurizes prior to material removal. .2m² to 3m² contaminated areas require local containment using HEPA cleaners.

Fungi in indoor air samples: Predominant indoor air fungi were: *Cladosporium herbarum*; *Moniliella sp.*; *Penicillium spp* with the specific identification of *P. citrinum*; *Fusarium spp*; *Rhizopus sp.*; *Aspergillus ochraceus*, *A. flavus*, *A. trichoderma*, *A. ustus*, *A. versicolor*, *A. syndowii*, *A. niger*; *Geotrichum candidum*. Ratios of >1 between indoor and outdoor concentrations requires further investigation (Singh, 1994). Table 1 shows hospital areas where indoor air fungal levels were > outdoor fungal levels. Semi-private hospital 1 had five out of nine areas with higher indoor fungal levels. Public hospital 2 then followed with four areas. Public hospital 1, semi-private 2 and private 2 had two areas. Only private 1 had no higher indoor levels.

Table 1. Ratios between indoor and outdoor air

	Public 1	Public 2	Semi-priv 1	Semi-priv 2	Private 1	Private 2
Pharmacy	<1	>1	<1	<1	<1	>1
ICU	<1	<1	<1	<1	<1	<1
Ward 1	>1	<1	<1	<1	<1	<1
Ward 2	<1	<1	<1	<1	<1	<1
Ward 3	<1	<1	>1	<1	<1	>1
Admin	=1	>1	>1	>1	<1	<1
Kitchen	>1	>1	>1	>1	<1	<1
CSSD	<1	>1	>1	<1	<1	<1
Theatre	<1	<1	>1	<1	<1	<1

Staff allergy: Not all 280 respondents were willing to submit to allergy skin prick tests. 123 (44%) were allergy tested: 10% were sensitized to mold with an additional 2% reacting positively to the *Penicillium* mold mixture, dust mite mixture elicited the highest allergy reaction (18%) and 1.6% reacted positively to cockroach allergens. Allergy questionnaire survey results of 123 “allergy” respondents: 17% reported asthma. 67% reported responses to pollutants such as smoke, fumes (perfumes, paints), dust or mold and also cold air. 26% missed work because of chest tightness/cough/wheezing/ prolonged shortness of breath. Upper respiratory tract allergy reactions: hay fever or allergic rhinitis 37%, diagnosed sinus 37%, itchy eyes 55%, itchy ears 36%, itchy roof of mouth 33% and itchy throat 41%.

Allergy test verses the allergy questionnaire: Chi-square tests indicated no association between the allergy questionnaire score with the allergy skin prick test results. Low false negatives and high false positives confirm the allergy questionnaire as an allergy screening tool ensuring a high percentage of the staff with potential allergies will be recognized. The skin prick tests are sensitive to specific allergens that may or may not be found in a particular work environment.

Respirable dust as clean room indicator: Particle count means were simplified by ranking. Lowest mean particle counts were represented by “1” with highest mean particle counts represented by “6”.

Table 2 indicates that private hospital 2 had the lowest overall particle score (1). Public hospital 2 scored (2). Higher scores were in the two semi-private hospitals with public hospital 1 having the highest score.

Carbon dioxide (CO₂) and comfort measurements: Maximum temperatures and carbon dioxide levels in the majority of hospitals showed that at times levels exceeded the applied standards. Relative humidity in all hospitals had maximums >70%. Air movement in most cases was within acceptable comfort limits.

Table 2. Ranking of hospitals and hospital section in terms of particle counts

	Public1	Public2	Semi-private1	Semi-private2	Private 1	Private 2
Pharmacy	4	3	5	6	2	1
ICU	6	2	4	5	1	3
Ward 1	6	2	5	3	4	1
Ward 2	5	1	4	6	3	2
Ward 3	5	2	1	6	4	3
Admin	6	2	5	4	3	1
Kitchen	6	2	1	5	3	4
CSSD	6	1	4	5	3	2
Theatre	3	6	5	4	1	2
Total score	47 (6)	21 (2)	34 (4)	44 (5)	24 (3)	19 (1)

Staff concerns regarding environmental comfort in 280 respondents: In all hospitals 20% or more of the respondents were concern regarding: warm temperatures, temperature variation, low air movement, relative humidity, stuffy air and smelly air. In private hospital 1 there were 8% concerned about temperatures being too warm. Overall assessments of sick building syndrome symptom potential in all hospitals were: lethargy (41.8%), blocked nose (25.4%), headaches (24.6%), dry throat (18.2%) and dry eyes (15%). Psychologically stressed Individuals may be more sensitive to environmental stressors (Berghlund, Gunnarsson, 1999)

Psychological stress: Demographic data were analyzed to establish whether significant differences exist between the three hospital types that could affect reported stress levels. Chi-Square tests suggest that there was not a great deal of difference between hospital groups. More than 50% of the 280 hospital staff were experiencing biopsychosocial stress (consisting of physical, psychological and behavioural stressors). Physical, psychological and behavioural sections of the SSCL were examined separately. Comparing the criterion of “3 or more” to the median of 4 for physical reactions and 3.5 for behavioural reactions in table 3, indicates

that more than 50% of the 280 respondents were experiencing stress. Psychological stress had lower significance. Extremely high maximums may indicate that respondents are not coping with stress.

Table 3. Physical, psychological and behavioural sections of the SSCL

Reactions	Median	Mean	SD	Maximum
Physical	4	4.6	3.7	17
Behavioural	3.5	6.6	8	21
Psychological	2	4.4	5	41.5

CONCLUSIONS AND IMPLICATIONS

Fungi were increased due to design problems, leakages, water damage and the poor ventilation system maintenance. Not all allergic reactions were caused by mold however, the synergistic impacts of the various stressors in hospitals warrants awareness and an effort to reduce stress sources. Presence of allergic staff is a concern and a broad based allergy questionnaire is a useful measurement tool to establish potential allergic staff. Hospital staff concerns included temperature, stuffy and odorous air. These conditions were related to the hot humid climate of Durban however; poorly ventilated environments and ineffective climatic controls exacerbated conditions. Particle counts were a useful means of assessing air cleanliness. However, each situation has to be investigated individually by maintenance staff with the support of health and safety personnel and management. The major influences of biopsychosocial stress in each hospital group with physical stress being the highest stressor.

ACKNOWLEDGEMENTS

The authors thank: Dr Maren Klich (United States Department of Agriculture, New Orleans) with fungal identification, the National Research Foundation and Medical Research Council for funding.

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VENTILATION EFFICIENCY, DAMPNESS AND MOLDINESS IN DURBAN DWELLINGS (SOUTH AFRICA)

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ABSTRACT

This is a study of 491 dwellings in a low-cost housing development in Durban, South Africa, to determine the prevalence of mold and dampness by means of a questionnaire survey and structured observations. About 51 % of the dwellings surveyed were found to be experiencing dampness ($> 3 \text{ m}^2$) and 46 % had visible surface molds, primarily on the walls (at least an average of 1 m^2). Predominant airborne fungal organisms identified included *Cladosporium*, *Aspergillus* and *Penicillium spp.* It was also found that 53 % of the dwellings lacked proper ventilation as airbricks were absent, which has the potential to impact adversely on the health of the occupants. The health issues of concern to the occupants were asthma (27 %); cough (25 %); sinusitis (25 %); chest infection (23 %) and rheumatoid arthritis (3 %). The results suggest that the occupants are exposed to dampness and fungal bioaerosols in their dwellings, which may be impacting on their health.

INDEX TERMS: Dampness, Mold, Questionnaire, Health

INTRODUCTION

In terms of Section 26 of the constitution of the Republic of South Africa, 1996, everyone has the right to have access to adequate housing, and the State must take reasonable legislative and other measures, within this framework the South African parliament recognizes that:

- Housing, as adequate shelter, fulfils a basic human need
- Housing, is both a product and a process
- Housing is a product of human endeavor and enterprise

- Housing is a vital part of integrated development planning
- Housing is a key sector of the national economy, and
- Housing is vital to the socio-economic well being of the nation.

With reference to the above it is evident that housing in South Africa is a compelling issue and a progressive process. The Waterloo, Housing development is one of the many governmental projects undertaken in an effort to eliminate the historical housing crises experienced, and also conforms to the housing delivery processes and other developmental programs, such as the Reconstruction and Development Program. Due to dramatic improvements in housing, with accompanying improvements in health and a decline in those diseases most clearly associated with lack of sanitation and overcrowding, interest in the social origins of ill-health had diminished, with particular neglect of the relationship between housing conditions and health. It is ironic that local authority housing, the so called “*mass housing*,” a movement which had as one of its most important motivation a desire to improve working-class health through better housing conditions, in recent decades produced the new urban slums, with implications for health risks.

This paper analyzes the quality of dwellings within the Waterloo Low-Cost Housing Development with reference to their design, occurrence of dampness, and the potential health implications.

METHODOLOGY

The list of developed sites within the program was used as a sampling frame, of which 491 dwelling units were visited and building/health questionnaires administered. Surface and sediment sampling with the aid of instrumentation was also conducted in these homes. Allowance was made for non-participation and withdrawals, during the research. Survey instruments were the Anderson single-stage air sampler, visual inspections, administration of questionnaires and the recording of temperature and relative humidity measurements.

Visual Inspection: After consent was obtained from the occupants, the houses were visually inspected for general appearance of the home, surface mold, water damage, dampness and ventilation.

Questionnaire survey: A questionnaire designed to gather information pertaining to the socio-demographics characteristics of occupant, the structural materials of the dwelling, occupant status, smoking habits, energy use, dampness, moldiness and health issues, were administered to 491 households. The questionnaire provided salient information on the dwelling, its size, materials used, occupancy, living conditions and health outcomes.

Mold Sampling: A total of 318 air samples were taken and analyzed, using an Anderson single stage sampler. There were 159 outdoor samples taken, and an equal number of indoor samples (56 from the Kitchen, 59 from the Bedroom and 44 from the lounge). The samples were taken under normal operating conditions at each home. The sampler deposited conidia onto malt extract agar (MEA) and dichloran glycerol 18 agar (DG 18) plates, as described in *Samson et al (1995)*.

RESULTS

The number of persons living in the 491 homes surveyed was 2414, of which 1019 were males and the remaining 1395 were females. There were 710 children under the age of 10. The occupants, which occupied a 4 roomed dwelling ranged from 1 to a maximum of 15. The average size per household was 5 occupants. There were 173 dwellings (35%) accommodating 6 or more occupants. The dwellings comprised of rooms ranging from 1 to 8, of which 47 % were one-room units, approximately 12 m² in size.

The Waterloo Development is fairly recent, and the age of the dwellings ranged from 1 to 6 years. The average age being 3 years. The nature of floor covering of the dwellings is presented in Table 1. Thirty-one per cent of the dwellings had no covering but a cement floor (grano) and 51 % had vinyl. A very minute group could afford carpets and tiles.

Table 1. Type of floor covering

PHASE	NO.-HOMES	CEMENT	VINYL	CARPETS	TILES
1	71	22 (31%)	29 (41%)	7 (10%)	12 (18%)
2	133	60 (46%)	42 (32%)	9 (7%)	18 (15%)
3	216	27 (12%)	157 (72%)	22 (10%)	11 (6%)
4	71	45 (63%)	22 (31%)	1 (1%)	4 (5%)

The dwellings were either owner or contractor built, but all were of brick and cement construction. The wall and its covering, plays an important role when assessing the potential for dampness. Table 2 summarizes the various wall coverings. In terms of ventilation, most of the occupants depend on natural ventilation for air circulation in their homes and they did not have air conditioning systems to keep their rooms cool during warm to hot days. The most reliable means of allowing air to enter the buildings were the airbricks, windows and doors. Although every dwelling was provided with at least one window and an entrance door, 53%

of the homes did not have airbricks. There were also situations where the only fitted window was not able to open, reducing the efficiency of adequate ventilation. The absence of airbricks, and poor ventilation, promotes condensation on building surfaces and subsequent mold growth.

Table 2. Type of wall covering

PHASE	NO COVERING	PLASTER ONLY	PAINT ONLY	PLAST& PAINT
1	12 (16%)	24 (34%)	8 (11%)	27 (39%)
2	31 (23%)	35 (26%)	16 (12%)	51 (39%)
3	12 (6%)	34 (16%)	113 (52%)	57 (26%)
4	4 (6%)	31 (44%)	20 (28%)	16 (22%)

The most common roofing material used in 276 dwellings were asbestos 56%, followed by Metal roofing sheets (39 %), and 24 dwelling (5%) with tiles. Ceilings were absent in 458 (93%) of the dwellings, Rhino boards were installed in 33 (7%) homes. The majority of the houses surveyed were damp (51.7 %) and 230 of the 249 damp homes (47 %) had a problem of indoor surface mold growth. Due to the lack of air bricks in 261 (53%) of the homes, majority of them being in Phase 3, adds to the reasoning as to why phase 3 is also the dampest phase (66%), and also most incidence of visible molds (47%).

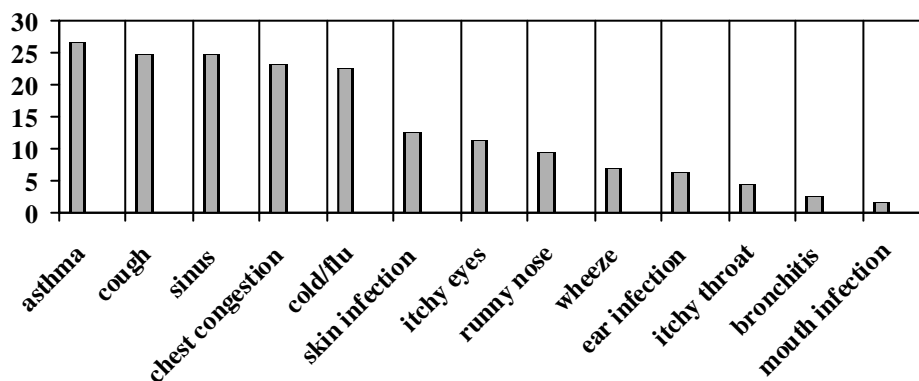
Visible molds were observed mainly on the walls (49%), followed by floors 17%. Seventy-two (31%) of the homes were damp and moldy at the areas where the walls and floors meet. This area was also prone to rising damp. Of the fungal bioaerosols sampled, there were various species identified as shown in Table 3. Generally the ratio of indoor: outdoor mold bioaerosols was approximately 1. This is not an ideal situation as it is suggestive of the indoor environment acting as a sink or a source of molds. *Cladosporium spp.* were the predominant mold followed by *Penicillium*, *Aspergillus*, *Acremonium* and *Fusarium spp.* respectively.

Table 3. Results of mold sampling

	<u>CFU ANALYSIS</u>			
	Kitchen	Bedroom	Lounge	Outdoors
n	56	59	44	159
CFU's	3472	5810	3089	11593
	CFU (%)			
<i>Acremonium</i>	220 (6%)	250 (4%)	180 (6%)	790 (7%)
<i>Aspergillus</i>	422 (12%)	660 (12%)	340 (11%)	1640 (14%)
<i>Fusarium</i>	250 (8%)	250 (4%)	180 (6%)	720 (6%)
<i>Cladosporium</i>	1845 (53%)	2610 (45%)	1440 (47%)	5920 (51%)
<i>Sterile mycelia</i>	310 (9%)	650 (11%)	325 (11%)	1080 (9%)
<i>Penicillium</i>	220 (6%)	90 (2%)	90 (3%)	520 (5%)
Unknown	205 (6%)	1300 (22%)	534 (16%)	923 (8%)

The most common symptoms expressed by the respondents are shown in Figure 1. With poor ventilation, dampness and mold growth in the dwellings studied, there were high cases of respiratory infections: asthma (27%; n=130), Cough (25%; n=122), sinuses (25%; n=121), chest infection (23%; n=113) and cold/Flu (23%; n=110). There were 29% of the dwellings where individuals smoked inside the house, a factor that may well exacerbate the poor respiratory health of occupants. The keeping of pets by 35 dwellers, did not indicate a significant negative impact on the health of the occupants. The use of wood, gas and kerosene by the householders, also did not indicate a significance negative health impact. Seventy per cent of the dwellings utilized electricity as their main source of energy and the remaining supplemented the electricity with wood, gas and paraffin, as cost effective measures.

Figure 1. Symptoms experienced by respondents



DISCUSSION AND CONCLUSION

Being a fairly new housing development (6 years old), one would expect minor problems, but this was not the case with this housing program. The poor workmanship in the buildings and the absence of moisture proofing, adds to the dampness and mold growth on the structures. According to Onguluna (1975), water can be introduced into the homes in several ways; rain, seasonal changes poor building design, defects and vandalism of the building. Dampness emanating from the ground is also a common problem in buildings that have poorly constructed foundations. In addition, water can be generated inside the building fabric by the different activities of the occupants such as: cooking, washing, watering indoor plants, pets, and the mechanisms used by the occupants to warm up the interior environment during cold and rainy seasons (Singh, 1994). It is therefore, not surprising that the most affected rooms in most of the moldy dwellings were the bathroom and kitchen with poor or no ventilation at all. It is within these enclosures that flushes of moisture can be generated during bathing and cooking.

The study indicates that a high percentage (46%) of the residential dwellings in the Waterloo Housing Development area have visible indoor surface molds. This is similar to observations made in two studies in South Africa which found that 42-46% of residential homes in Durban (Sekhatha *et al.* 2000) and Port Elizabeth (Thomas *et al.* 1999) were effected with dampness and mold growth. The conditions of moldiness on the walls and floors may be used to characterize individual house lifestyle of the occupants and their financial status. This may lead to the improper maintenance of their dwellings, and the results of surface mold growth. In terms of treatment of the mold, it was deduced that 48% of the homes had no

treatment strategy in place, maybe because most were unaware of the consequences that might be caused by the presence of indoor surface mold growth.

The homes that applied treatment used the less expensive method of treating indoor fungal growth, notably water, household detergents and paint. This would treat the consequence and not the cause of the mold growth in the buildings. These remedial actions were for aesthetic purposes, as there is no realization that molds are biological organisms. Most paints contain hydrocarbons products that might provide suitable nutrition for the survival of the mold, therefore, painting the affected area may temporarily solve the problem if not permanently aggravate it. The ideal way of reducing the growth is to use mold-free material or fungicides that will not create any secondary complications (Singh, 1994). However, these measures (replacing building material or improving ventilation) are not favored because of cost constraints.

In view of the findings in the study, it is necessary that educational programs be developed with the contributions of the relevant stakeholders in order to increase community awareness about dampness and mold growth. Improved workmanship and selection of materials on the builder's part is also recommended. There is also a need to ensure that communities take the necessary precautionary steps to live healthier. In this regard it is imperative that laws and regulations be formulated at national and international level to assist the communities in promoting healthy living.

ACKNOWLEDGEMENTS

We wish to thank the Medical Research Council, National Research Foundation, and the University of KwaZulu Natal Research Fund for financial assistance.

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MICROBIAL EXPOSURE IN SCHOOLS STUDIED BY CHEMICAL MARKER ANALYSIS. COMPARISON BETWEEN THREE DIFFERENT COUNTRIES

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ABSTRACT

We used gas chromatography–tandem mass spectrometry to analyze microbial components in 85 samples of airborne dust from schools in Jordan, Sweden, and Poland during both summer and winter. In each country, we conducted such sampling in two schools: one in urban and the other in rural. The microbial marker profiles differed significantly between the schools and seasons. For example, samples from Jordan contained low levels of ergosterol and high levels of 3–OHFAs of 10, 12, and 14 carbon chain lengths relative to such acids of 16 and 18 carbons in comparison with samples from Sweden and Poland. This dissimilarity in 3–OHFA distribution indicates significant differences in the populations of Gram–negative bacteria. Moreover, muramic acid exhibited the smallest variation between schools and seasons. Results demonstrate that exposure to microorganisms in indoor air in school buildings may differ markedly between countries, seasons, urban and rural environments.

INDEX TERMS: GC–MS/MS, House Dust.

INTRODUCTION

We do not yet fully understand how humans are affected by exposure to microorganisms in different environments. People in the world spend 80% or more of their time indoors, therefore the microbial populations inside buildings may have the greatest impact on human well–being. Research has shown that levels of bacterial endotoxin in the domestic environment are positively correlated with the severity of asthma. Scientists also suggested that exposure to microorganisms at an

early age may protect against allergies. Lipid A is a component of LPS that contains 3-OHFs, which can serve as chemical markers to measure total LPS. Similarly, muramic acid is used to measure peptidoglycan and bacterial biomass, whereas ergosterol represents a marker of fungal biomass. In the present study we use chemical marker analysis to characterize the microbial exposure in schools in Jordan, Poland and Sweden.

MATERIALS AND METHODS

Two schools were chosen in each country: one in a city, and the other in a village. Dust samples were collected using plexiglass plates (0.5 x 0.5 m) hanging from the ceiling for four weeks in four different rooms in each school in both winter and summer. In addition, one plate was placed immediately outside each school for collection of outdoor samples.

Ergosterol was determined according to Saraf *et al.* (1999). Briefly, dust (7–10 mg) was heated in 1 ml of 10% methanolic KOH (80°C, 90min). After cooling, 5 µl of dehydrocholesterol (internal standard) was added, and the preparation was purified and subjected to trimethylsilyl (TMS) derivatization. Muramic acid and 3-OHFs were determined by GC/MSMS according to Bal and Larsson (2000) and Saraf *et al.* (1999).

All data were analyzed by SPSS for Windows 11.1. The comparisons of the three countries were done by one-way analysis of variance (ANOVA), and the results obtained were further analyzed by Tukey multiple range tests. The comparisons of city and village samples, and of indoor and outdoor samples, were done using two-tailed Student's t-test. The comparisons of summer and winter samples were analyzed by paired t-test. When the data exhibited a skewed distribution, we performed log₁₀ or square root transformations of the basic variables before the statistical analysis. Non-parametric test (Mann-Whitney) was applied when the data transformations failed to achieve near-normal distribution. Statistical significance was defined as $p < 0.05$.

RESULTS

The concentrations of ergosterol varied between 359–37219 pg/mg of dust. Table 1 shows the mean concentrations found in Jordan, Poland and Sweden. Sweden showed the highest concentrations of ergosterol, both indoors and outdoors, and Jordan showed, by far, the lowest concentrations. In Jordan, the concentration of ergosterol in indoor samples constituted 74% of outdoor concentration, in Poland the corresponding figure was 11% and in Sweden 17%.

Table 1. Concentrations of ergosterol (pg/mg dust, average of all samples collected) in the studied countries

	Jordan	Poland	Sweden
Indoor	505	1899	3862
Outdoor	679	17558	22315

The concentrations of MuAc varied between 8.1 and 115 ng/mg. Poland showed the highest concentrations, in both indoor and outdoor samples, followed by Sweden and Jordan, and the difference was most pronounced for the outdoor samples (Table 2). The MuAc concentrations in indoor samples constituted 95%, 39% and 84% of outdoor concentration in Jordan, Poland and in Sweden respectively.

Table 2. Concentrations of MuAc (ng/mg dust, average of all samples collected) in the studied countries

	Jordan	Poland	Sweden
Indoor	14.9	20.2	19.3
Outdoor	15.6	51.4	23

Finally, the 3-OHFA concentrations varied between 13.1 and 66.5 pmol LPS/mg dust. Jordan showed the highest concentration of 3-OHFAs in indoor dust samples followed by Sweden and Poland (Table 3), and Sweden showed the highest concentrations in the outdoor samples followed by Poland and Jordan. The 3-OHFA concentration in indoor dust constituted 80% of the outdoor dust concentration in Poland, and 72% in Sweden. In Jordan, the 3-OHFA concentrations were higher indoors than outdoors.

Table 3. Concentrations of LPS (pmol/mg dust, average of all samples collected) in the studied countries

	Jordan	Poland	Sweden
Indoor	48.2	31.4	34.4
Outdoor	25.8	39.4	47.8

Interestingly, indoor samples - unlike outdoor samples - from Jordan contained remarkably high levels of 3-OHFAs of 10, 12, and 14 carbon chain lengths relative to such acids of 16 and 18 carbons in comparison with samples from Sweden and Poland indicating significant differences in the populations of Gram-negative bacteria (Table 4, Figure 1 and 2).

Table 4. Significant differences in 3-OHFAs (C10–C18) concentrations between the dust samples from the three countries (ANOVA Tukey test).

Sample	Season	Location	Marker analyzed	Jordan	Poland	Sweden	Signif.
Indoor	Summer	City	C10	28	12	0.0	<i>b*</i>
			C12	54	33	10	<i>b*</i>
	Winter	City	C10	25	0.4	0.0	<i>a**,b**</i>
			C12	51	7	11	<i>a**,b**</i>
			C14	36	10	21	<i>a*</i>
			C16	38	22	50	<i>c*</i>
			C18	23	10	30	<i>c*</i>
					Village	C10	34
			C12	57	15	15	<i>a**,b**</i>
			C14	48	15	31	<i>a*</i>
			C18	13	19	88	<i>b*,c*</i>
Outdoor	Summer	City	C12	5.6	15	68	<i>b*</i>

* $P \leq 0.05$, ** $P \leq 0.01$; Symbols: a, Jordan/Poland; b, Jordan/Sweden; c, Sweden/Poland; C10–C18, 3-OH fatty acids with the indicated number of carbon atoms. Numbers refer to the mean concentration of the four collected samples.

Figure 1. Distribution of 3-OHFAs (pmol/mg) in indoor dust samples collected in summer and winter in rural and urban schools in Jordan, Poland, and Sweden. The illustrated data represent mean concentrations.

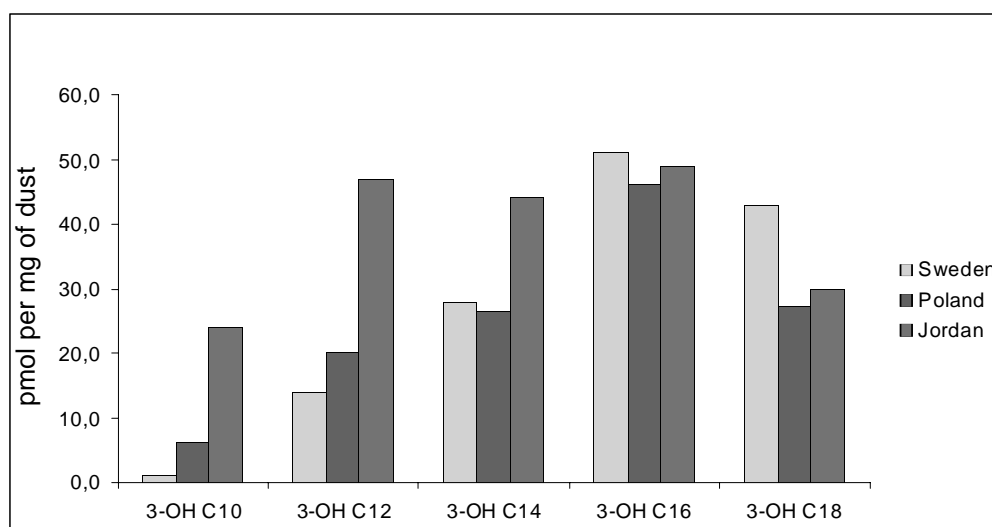
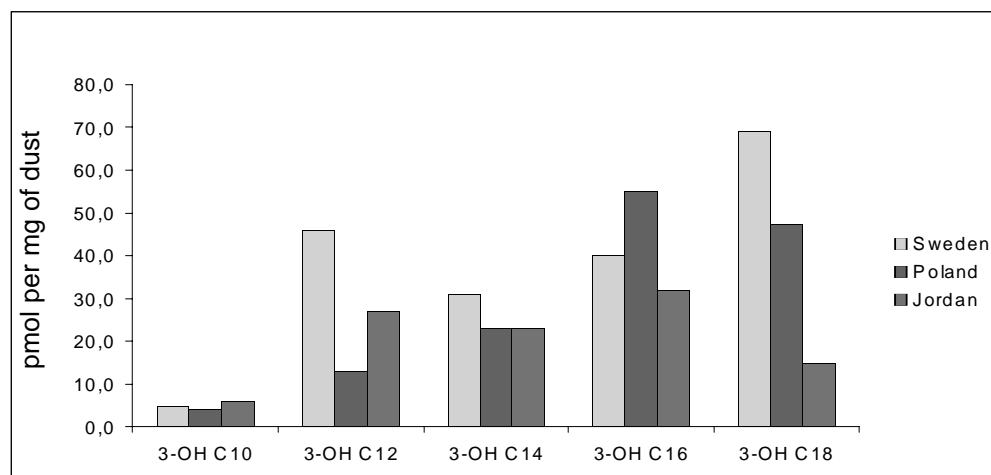


Figure 2. Distribution of 3-OHFAs (pmol/mg) in outdoor dust samples collected in summer and winter immediately outside school buildings located in rural and urban parts of Jordan, Poland, and Sweden. The data shown represent mean concentrations.



DISCUSSION

Results published in the literature suggest that our well-being is affected by exposure to the microorganisms in our surroundings. However, we are far from having a complete understanding of the complex interactions between microbes and health implications. One explanation is that, until recently, it has been very difficult to study non-culturable microorganisms. However, like culturable microbes, the non-culturable (and non-viable) counterparts contain a range of toxic compounds. Peptidoglycan and lipopolysaccharide are two prominent components of the bacterial cell wall. Studies have shown that peptidoglycan is toxic to hamster tracheal epithelium *in vitro* (Cookson *et al.* 1989). Levels of muramic acid in swine-house dust have been correlated with granulocyte counts and body temperature in humans who inhaled the dust (Zhiping *et al.* 1996). Other investigators have reported that dusty indoor environments in schools are associated with adverse health effects and that elevated levels of endotoxin in household are connected with increased severity of asthma. Endotoxin exposure at home may independently increase the risk of wheezing in infants (Park *et al.* 2001), and sensitization to molds may be a risk factor for asthma in adults (Zureik *et al.* 2002)

Our previous studies on chemical markers were conducted in the United States and Sweden and we found that the relative amounts of the different 3-OHFAs in indoor environments varied to some extent depending on the type and location of the sampling sites in the building (Hines *et al.* 2000; Saraf *et al.* 1997). In comparison, the marker profiles found in the present study differed much more dramatically. For example, compared to the dust from Sweden and Poland, samples from Jordan contained substantially lower levels of ergosterol (per milligram of dust) and considerably larger relative amounts of the shorter-chain 3-OHFAs compared with the longer-chain 3-OHFAs (Table 4). Notably, the shorter 3-OHFAs correlate better with bioactivity (*Limulus* assay) than the longer 3-OHFAs do. The wide variation that we observed in levels of microbial markers may have been due to climatic factors, for example temperature and humidity, and also to differences e.g. in aspects such as the way the school buildings were constructed, cleaned, and ventilated, as well as the number of children active in the studied rooms and the lifestyles. Taken together, our results indicate that the children in the studied schools are exposed to highly different microbial communities. Further research is needed to explain how the indoor exposure to microorganisms—as indicated by patterns of analyzed microbial markers—affects our health and quality of life.

ACKNOWLEDGEMENTS

Financial support from FORMAS (Sweden) is gratefully acknowledged.

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FUNGI SURVEYS IN TROPICAL SOUTHEASTERN BRAZIL

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ABSTRACT

In December 96, the Manguinhos Library in Rio de Janeiro, Brazil, the largest biomedical reference library in Latin America, was closed to the public because of severe fungi infestation due to problems associated with the air conditioning units. The source of moisture was mainly elevated indoor relative humidity. Preliminary measurements of fungi levels in January, 1997, before remedial work showed airborne fungi levels ranged from 600 to 960 cfu/m³. Indoor air temperatures in the offices were 28-32 °C and relative humidity was 72-84 % at the time of intervention. Follow-up measurements taken after 6 months showed a 70% drop in fungi concentration.

Following up the Manguinhos library episode, bioaerosol measurements were carried out in several buildings within the Fiocruz campus. Levels up to 893 cfu/m³ of total fungi were found in a nursery school room.

INDEX TERMS: Fungi, Tropical climate, Moisture, Brazil, Rio de Janeiro

INTRODUCTION

Fungi are ubiquitous in both indoor and outdoor environments. It is well established that the concentrations of microorganisms in urban air are strongly related to the local weather conditions such as humidity, temperature, and wind speed. In Southeastern Brazilian hot and damp summer weather, high relative humidity and surface condensation can potentially contribute to the presence of fungal growth indoors. Indoor environmental research conducted in Brazil has been very limited (Costa, Brickus, 2000) and the participation of health professionals and comprehensive research studies is conspicuously lacking. At the moment, scarce information is available on indoor and outdoor pollutants in Brazil (Brickus *et al.* 1998). In spite of the fact that a significant part of the Brazilian population spends most of

their time inside buildings, the indoor environmental issue has been relatively overlooked. Moreover, the percentage of Brazilian buildings with indoor air quality problems has not been established.

This paper presents results from two distinct surveys within the Fiocruz campus: The Manguinhos Library, and Healthy Fiocruz. The present work aims to contribute to the growing volume of IAQ references in Brazil.

METHODS

Geographic characteristics: The climate in Rio de Janeiro is tropical with hot and humid summers and drier winters. The monthly average temperatures vary between 24°C (June/July-winter) and 33°C (January/February-summer). Situated at a latitude of 22°54'S, and a longitude of 43°10'W, the city experiences a climate with uniformly high yearly temperatures, high humidity and abundant rainfall averaging 2381 mm y⁻¹.

Site Descriptions: *The Manguinhos Library:* The new Manguinhos library building was inaugurated in August 1995. In December 1996 a severe mold growth incident occurred in the building during the holiday vacation when the building was not occupied. The building is a two-story building with an area of 5,600 square meters (m²). The interior building material consists of concrete walls and ceiling and has a tile floor throughout. The building has central air conditioning with 4 air-handling units per floor and ducts in the ceiling cavities. There is an open garden in the middle of the building. A total of six sampling points were selected in the library building. Airborne fungal spores were sampled in January 1997 and July 1997.

Healthy Fiocruz: The Fiocruz campus location is on an area of 820,000 m² in the North Zone of Rio. It is a huge green area in the midst of a heavily inhabited region containing impoverished communities and a large number of small businesses. The following units were investigated in this study: *INCQS* - The National Institute for Quality Control of Health is located in this building. It has a central air-conditioning system. Airborne samples were taken in the library, auditorium, clean room, office room and in the laboratories of Microbiology and Chemistry. *Nursery School* - This building has wall air-conditioning and at the time of the investigation, the majority of rooms had open doors and windows creating a naturally ventilated environment. *Castle* - This office building is a historic Moorish building with wall air-conditioning units. Samplings were carried out during a one-week period in February, 1998.

Sampling and analysis: Sampling for airborne viable fungi was made by using a six-stage cascade impactor (Andersen Sampler, AMS). Sabouraud dextrose agar

was used as a general screening medium. Indoor air sampling was performed at a central point in each room approximately 1.0 m above the floor. The outdoor air was sampled at the roof of each building near the outdoor air intake. Samples were collected for 15 minutes at 28.3 L/min during normal occupancy hours. Cultures on Sabouraud were incubated for 5.8 days at 27°C. Fungi were identified by colony characteristics and microscopic examination with lactophenol blue stain.

RESULTS

The indoor environment approach began with a severe fungi infestation at the Manguinhos Library because of chronic problems within the central air conditioning system. In January, 1997 during the first walk-through, when the library was closed to the public and staff, extensive visible fungi growth was clearly noticed throughout the interior surfaces of the building, including books, bookshelves, desks, computers, chairs, artworks, window glass, walls and the ceiling. Shortly after the detection of the fungi infestation, the indoor readings (January, 97-summer season) indicated that the indoor temperature ranged between 28-32 °C and relative humidity ranged between 72-84 %, whereas the outdoor temperatures ranged between 24-32 °C and relative humidity ranged between 70-77 % (Figure 1). The temperature and relative humidity readings taken in June (winter season) indicated a continuing need to improve the balance of the air conditioning system to control indoor temperatures and relative humidity.

Figure 1(A) Variation of temperature during the fungi infestation at the Manguinhos Library.

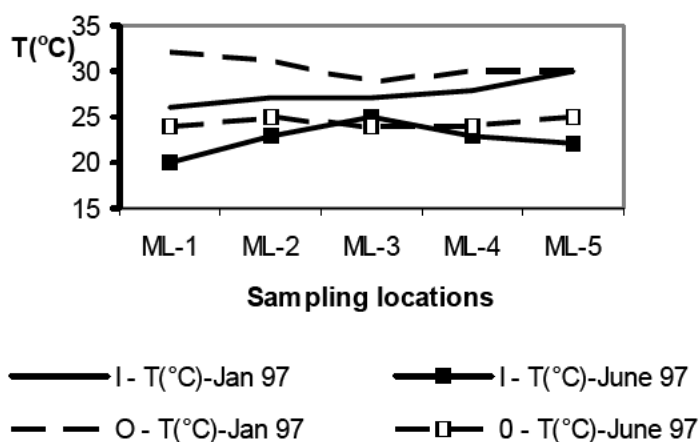
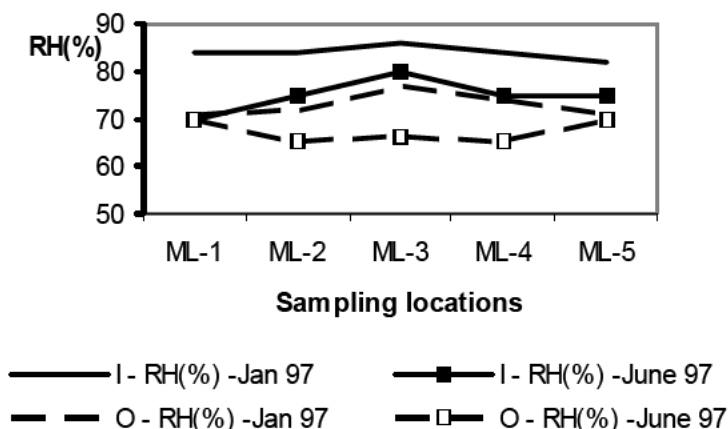


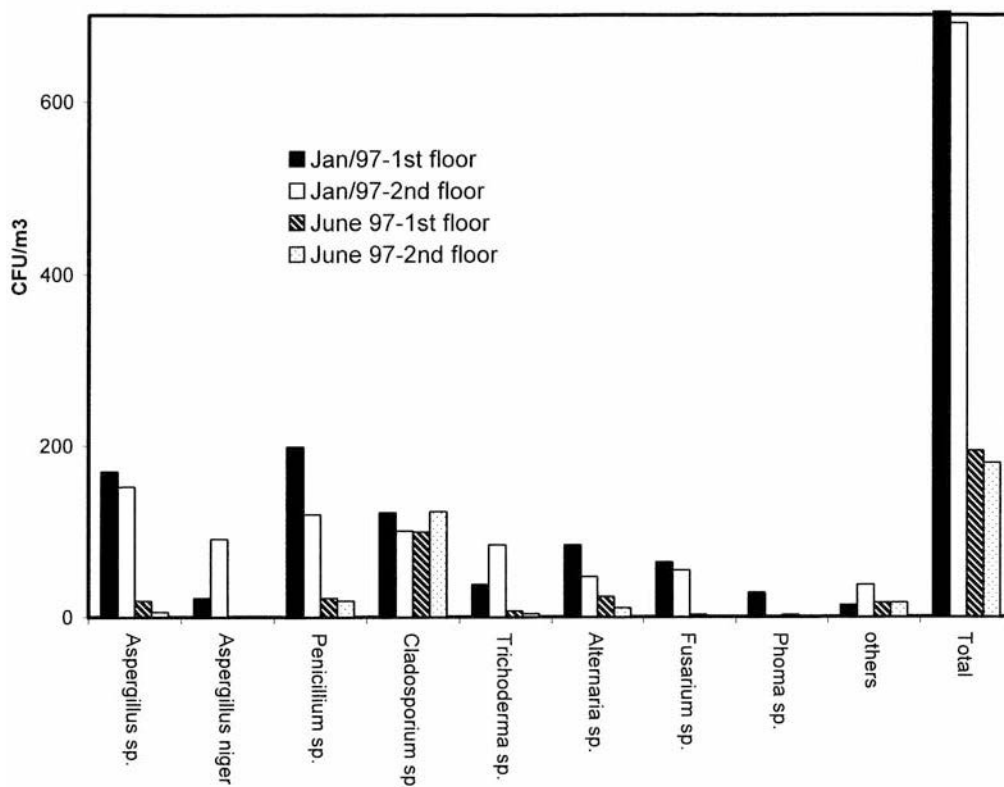
Figure 1(B) Variation of relative humidity during the fungi infestation at the Manguinhos Library.



The following remedial work was performed: installation of gutters to minimize water incursion from the exterior walls; isolation of the interior garden with glass walls and waterproof of the ground around the building and within the interior garden in order to minimize moisture; wet wiping and/or HEPA vacuuming the building envelope and its contents. The books were individually HEPA vacuumed and dry wiped under a hood chamber. An art restoration expert restored the artwork. Dehumidification equipment was installed throughout the building to reduce relative humidity to levels lower than 50%. During the evaluation of the library it was discovered that the outdoor air intakes were located at the floor level near the planter. The air intakes were redirected to the roof.

Several genera of fungi were identified at the library during the infestation. Figure 2 shows the average distribution of the total and specific genera identified in the first and second floors before (January, 97) and after (June, 97) the remediation. In January, 97, before the remedial work, the most common genera/types observed were *Aspergillus sp.* (96-164 cfu/m³), *Aspergillus niger* (68-154 cfu/m³) and *Penicillium sp.* (79-246 cfu/m³). Other molds made only a moderate contribution. In the June 97 sampling, after the remedial work, the total fungi levels dropped by 70%. The most common genus detected was *Cladosporium sp.* (62-183 cfu/m³), whereas *Aspergillus sp.* (12-22 cfu/m³), *Aspergillus niger* (not detected) and *Penicillium sp.* (14-26 cfu/m³) levels were significantly lower than before the remedial work. Figure 2. Average distribution of total and identified fungi (cfu/m³) at the library before (January, 97, n= 12 samples) and after (June, 97, n= 12 samples) remediation.

Figure 2 Average distribution of total and identified fungi (cfu/m³) at the library before (January, 97, n= 12 samples) and after (June, 97, n= 12 samples) remediation.



In regards to the Healthy Fiocruz study, three units at the Fiocruz campus were evaluated. Results for average indoor and all outdoor samples collected during this survey are shown in Table 1. Six indoor locations at the *INCQS* building were investigated. Total average viable fungal levels ranged from 69-550 cfu/m³ indoors in the *INCQS*. The most prevalent fungi identified in this site were *Aspergillus sp.* (7-238 cfu/m³), *Cladosporium sp.* (14-150 cfu/m³) and *Penicillium sp.* (7-120 cfu/m³). The remaining fungi made a moderate to substantial contribution to the total levels.

Levels up to 893 cfu/m³ of total fungi were found in the purple room (for 4-5 year-old children) in the nursery school. Very low levels of fungi were found in the two Castle sampling locations investigated. The genera *Alternaria sp.*, *Neurospora sp.* and *Aspergillus sp.* were predominant in outdoor air.

Table 1. Average of total fungi levels in the Healthy Fiocruz Study

INCQS	Total Fungi	Nursery School	Total Fungi	Castle	Total Fungi	Outdoor	Total Fungi
Auditorium	467	Green Room	6570	Room 3	67	INCQS	91
Library	550	Purple Room	893	Room 7	48	Av Brazil	210
Office	69	Sleeping room	217			Castle	134
Chem. Lab.	324						
Microb. Lab.	147						

DISCUSSION

This evaluation in the Mangunhos library showed how molds can become an IAQ problem when moisture and the presence of nutrients occur in the building. Two months after opening this library, mild fungal growth was noticed in books. The management tried to keep the relative humidity low by decreasing the indoor temperature, which created thermal discomfort for the staff. To make things worse, the air conditioning (AC) system would be shut down at night and over the weekend to save energy. The oscillation of temperature and relative humidity indoors favors microbial deterioration of the books within the building.

During the Holiday season of 96/97, the AC was not operational; the indoor environment became very humid and hot because the typical regional season. This situation created optimal conditions for fungal growth to spread throughout the building's interior surfaces. As a consequence of this problem, the library faced a severe fungi infestation, with a high percentage of the staff complaining about adverse health effects (Strausz, 2001), which resulted in the closing of this facility in January, 97.

When the genera *Penicillium sp.* and *Aspergillus sp.* were calculated to be dominant in all samples. Exposures to species of *Penicillium* and *Aspergillus* have been associated with occupational asthma and hypersensitive pneumonitis (Grammer, Patterson, 1982). A comparison between levels of fungi genera at different periods of sampling revealed particular differences. Levels up to 164 cfu/m³ (*Aspergillus sp.*), 153 cfu/m³ (*Aspergillus niger*) and 246 cfu/m³ (*Penicillium sp.*) were found in January, 1997. On the other hand, six months after this episode, the levels were less than 30

cfu/m³ collectively of genus *Aspergillus* and *Penicillium* fungi, indicating the environmental fungi were under control.

The Manguinhos library experience created awareness in the Fiocruz campus regarding IAQ issues. Environmental questions were raised from different parts of the campus and an exploratory investigation was designed for some units at the campus. The *INCQS* was selected because the building has a central air conditioning system and a wide diversity of occupational activities, including Chemistry and Microbiology laboratories, several administrative offices, a library, an auditorium, and so on. The lowest levels of airborne fungi measured were in the clean room, which was expected because of HEPA filters in the AC-system. The highest levels of fungi were found in the library and the auditorium, most likely because of the presence of books and carpeting, respectively.

Total levels of fungi were the highest at the castle site, but these were phylloplane species. The Nursery School showed the highest values of total fungi within the campus. The rooms evaluated were under natural ventilation, except in the sleeping room (equipped with a window AC unit). These high values appear to be related to the contribution from vegetation around the building. Conversely, the levels of total fungi were lowest at the Castle site, in spite of complaints from the staff regarding the “musty” smell within the evaluated offices. The majority of staff reported symptoms such as eye and nose irritation, and dry throat.

CONCLUSIONS AND IMPLICATIONS

Several years after the mold infestation event, the library administration has retained a different AC maintenance company, which redesigned the air balance within the library. However, the library still deals with staff complaints regarding thermal discomfort. The readjustment of the air balance might not be a sufficient way to solve the problem. Proper dehumidification of the air should be considered. The Manguinhos library is now considered a consulting library for unraveling indoor air quality problems in this particular environment in Brazil.

Much more research is needed to characterize airborne fungi in Brazil. The type of climate, topography, vegetation and specific construction practices may play a major role in fungal exposure to the general population.

ACKNOWLEDGMENTS

This study was financially supported by the Ministry of Health - Fundação Oswaldo Cruz. Dr L S R Brickus thanks CNPq/Rhae and Fiocruz/Faperj for a research fellowship. Dr J C Moreira thanks CNPq for financial support.

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Chapter 10

Remediation

Session chairs:

Phil Morey, Ken Ruest

THE EFFECT OF CONSTRUCTION SITE DUST ON MOLD CLEARANCE SAMPLING

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ABSTRACT

Air sampling for fungi was conducted at three buildings where new construction or build-back activities disturbed the soil. Data analysis indicated that air samples collected near soil disturbed by construction activities contained elevated levels of soil fungi including *Penicillium* and *Aspergillus* species. Depressurized containments constructed around scaffolding can act as a sink for aerosolized soil fungi. Clearance samples used to judge the success of mold remediation may be difficult to interpret due to elevated levels of entrained soil fungi. Mold remediation clearance criteria for scaffold containments near construction activities should focus primarily on a visual inspection that ensures moldy materials (and dusts generated during remediation) have been removed. Clearance air samples that may be collected in a scaffold containment should be accompanied by outdoor samples that represent the air that is flowing into a depressurized containment.

INDEX TERMS: Fungi, Construction site dust, Mold clearance sampling, Mold remediation

INTRODUCTION

Clearance sampling for airborne fungal spores is often utilized in mold remediation projects. The objective of clearance air sampling is to determine if the rank order (kinds) of fungal spores after clean-up in the affected building is similar to the rank order of fungal spores in the outdoor air. Thus, phylloplane fungi, which normally dominate the outdoor mycoflora, should dominate the indoor air in the remediated building if clean-up has been accomplished appropriately. *Penicillium* and *Aspergillus* species are normally present in soil (these fungi degrade organic material in the soil) and are often aerosolized during soil movement and trenching operations (Streifel, 1988).

Exterior depressurized containments may be constructed around scaffolding when moldy building envelope materials such as exterior sheathing are dismantled and removed. These depressurized containments are used to keep fungal spores dislodged during remediation from entering interior or occupied areas. The purpose of this study was to determine if soil fungi from outdoor construction could enter exterior containments and potentially affect the interpretation of clearance air sampling data.

METHODS

This study was carried out in three U.S.A. buildings where sampling for fungal spores occurred coincidentally with build-back or new construction activities. Soil disturbances at these buildings occurred simultaneously with sampling. Air and settled dust samples collected in study building 1 were cultured on malt extract (MEA; 2% malt without dextrose; [AIHA, 1996]) and dichloran 18% glycerol (DG-18; [AIHA, 1996]) agars incubated at 25°C. Air sampling was carried out using a Surface Air System (SAS [International PBI, Milan, Italy]) portable culture plate impactor operating at a flow rate of approximately 0.18m³/min. Samples were incubated for at least 10 days before fungal colonies were identified using standard mycological criteria. Air samples in study buildings 2 and 3 were collected using only a Burkard personal volumetric sampler (Burkard Manufacturing Co. LTD, Rickmansworth, England) operated at 0.01m³/min.

RESULTS

Study Building 1: Settled dust and air samples for culturable fungi were collected from a low rise government/educational building located in the coastal Washington state area of the U.S. Environmental Protection Agency - Building Assessment Survey and Evaluation (US EPA-BASE) Region I (Womble *et al.* 1996). While the building was being renovated, dusts and soil associated with outdoor construction activities were being tracked into the building.

Table 1. Culturable fungi present in construction dusts from Building 1.

Dilution Plating Media	PEN	CLA	OTHER FUNGI
	Mean number of colonies on plate		
MEA	36 (53%)	7 (10%)	25 (37%)
DG-18	45 (53%)	22 (26%)	18 (21%)

CLA = *Cladosporium*, PEN = *Penicillium sp.*; No. of samples = 6 for each medium; *Penicillium* not speciated

Dusts were collected from floors and were dilution plated on MEA and DG-18 agars. On both media, *Penicillium spp.* accounted for 53% of all fungi colonies identified from floor dusts. Table 1 summarizes data from 12 dilution plated samples.

Air samples for culturable fungi in Building 1 were collected indoors near sites where floor dust samples were collected (indoor construction activities were still underway) as well as in the outdoor air (Table 2). *Penicillium spp.* accounted for, on average, 79% of total colonies identified in indoor air samples. In samples collected outdoors away from construction activities, *Penicillium spp.* accounted for only 8% of total colonies. The number of colony forming units per cubic meter (CFU/m³) among samples collected indoors was substantially greater than in samples collected outdoors. Table 2 summarizes data from 30 air samples for culturable fungi.

Table 2. The concentration of culturable airborne fungi present indoors and outdoors at Building 1.

Sample media and location	PEN	CLA	TOTAL
	CFU/m ³		
MEA, indoors	321	42	419
MEA, outdoors	6	37	95
DG-18, indoors	316	29	386
DG-18, outdoors	9	14	84

CLA = *Cladosporium*, PEN = *Penicillium spp.*; Indoor sites (near construction activity), No. of samples = 6 for each medium; Outdoor sites (on roof or on grade >30m from building), No. of samples = 9 for each medium; CFU/m³ = colony forming units per cubic meter of air

Study Building 2: A mid-rise government/educational building (Building 2) in the coastal California region of US EPA-BASE Region J underwent mold remediation. The building exterior was contained and negatively pressurized during removal of the envelope, including moldy construction materials. Reconstruction of the envelope occurred in phases along building elevations as soon as mold remediation was completed in each phase.

Table 3 summarizes outdoor spore trap sampling data near Building 2 (near soil disturbances) and at remote sites upwind (>50m) of the building. Over a 20 day period, 59 spore trap samples were collected. On average, the concentration of *Penicillium/Aspergillus* was 162 spores per cubic meter of air (spores/m³) (17% of

the total) in samples collected upwind/away from the building. The average concentration of *Penicillium/Aspergillus* was 986 spores/m³ (53% of total) near the building during build-back activities. *Penicillium/Aspergillus* levels in samples collected near the building were significantly greater than (p=0.01) those collected upwind/away from the building. The mean total spore concentration in outdoor air near the building was approximately twice that of samples collected upwind/away from the building. In Table 3, the data collectively shows that *Penicillium/Aspergillus* dominated the fungi in outdoor air near the building during construction activities.

Table 3. The concentrations of airborne spores collected outdoors at sites remote (>50m) and close (<10m) to Building 2 which was undergoing envelope reconstruction.

Sample Location	PA	CLA	TOTAL
	Spores/m ³		
Upwind of (>50m) building	162a	454	921
Near (<10m) building undergoing construction	986a	597	1,866

CLA = *Cladosporium*; PA = *Penicillium* + *Aspergillus*; Upwind samples, No. of samples = 34; Near building samples, No. of samples = 25

^a Mann-Whitney sum of ranks test rejects the hypothesis that there is no statistically significant difference between the mean values of PA upwind of the building and near the building at p=0.01.

Study Building 3: A low rise government/educational building (Building 3) in the coastal California region of US EPA-BASE Region J underwent mold remediation in order to remove envelope building materials that were colonized by molds. Depressurized exterior containments built around scaffolds were used to keep dusts generated during demolition from entering the building interior. Three-chamber decontamination units were the only entrances into the scaffold containments. The decontamination unit was also used as a portal to remove contaminated building materials from the scaffold containments.

During the mold remediation, nearby construction activities (landscape removal, trenching operations, heavy equipment removal, stucco application) were ongoing. Table 4 summarizes analytical results from approximately 300 spore trap samples collected from locations in and around Building 3.

Upon completion of remediation activities in a scaffold containment, clean-up effectiveness was first verified by visual inspection. The remaining envelope construction materials were inspected to assure that all moldy materials (and dusts generated during material demolition) were removed from the containment. After an acceptable visual inspection, spore trap samples were collected in order to further judge the effectiveness of clean-up (Table 4). Samples collected inside the depressurized scaffold containments were consistently dominated by *Penicillium/Aspergillus*. On average, samples collected within the scaffold containments contained 38,143 spores/m³ *Penicillium/Aspergillus* (70.7% of total). *Penicillium/Aspergillus* levels outdoors close (<10m) to the building envelope averaged 894 spores/m³. At more remote outdoor sites, *Penicillium/Aspergillus* levels were only 119 spores/m³. Both *Penicillium/Aspergillus* levels within the scaffold containment and outdoors <10m from the building were found to be significantly greater than (p=0.01) levels in the outdoors >50m from the building (Table 4). This data suggests that a substantial amount of fine dust remained on rough (wood planking) and on inaccessible surfaces of scaffolding. The data from Building 3 suggest that soil fungi aerosolized during various construction activities can become entrained into scaffold containments. Air and dust enter the scaffold containment through the decontamination unit used to access the scaffold.

Since air clearance could not be achieved, scaffold surfaces were re-cleaned and the scaffold containment was gradually disassembled allowing outdoor air to “wash” exposed scaffold and envelope surfaces. Once scaffold containments were no longer depressurized as compared to the outdoor air, *Penicillium/Aspergillus* concentrations eventually became approximately equal to the surrounding outdoor air concentrations.

Penicillium/Aspergillus concentrations within interior containments in Building 3 (127 spores/m³) were approximately equal to *Penicillium/Aspergillus* levels (119 spores/m³) in the outdoor air at locations 50m or further away (Table 4). The decontamination units for all interior containments had no direct air intake from outdoor air potentially contaminated by construction site soil disturbance activity.

Table 4. Average concentrations of airborne spores collected indoors and outdoors at Building 3 which was undergoing simultaneous mold remediation and build-back.

Sample Location	PA	CLA	TOTAL
	Spores/m ³		
Outdoors, >50m from building	119a	1,535	2,437
Outdoors, <10m from building	894a	17,475	22,450
Indoors, within interior containments	127	80	454
Within scaffold containments	38,143a	7,402	53,938

CLA = *Cladosporium*, PA = *Penicillium* + *Aspergillus*; Outdoors, >50m from building, No. of samples = 65; Outdoors, <10m from building, No. of samples = 60; Indoors, within interior containments, No. of samples = 86; Within scaffold containments, No. of samples = 94

* Mann-Whitney sum of ranks test comparison of spores/m³ *Penicillium*/*Aspergillus*:
 Outdoors,>50m from building = Within scaffold containment, reject at p=0.01
 Outdoors,>50m from building = Outdoors, <10m from building, reject at p=0.01

DISCUSSION

Outdoor construction at buildings almost always results in soil disturbances. The following activities were noted as causing soil aerosolization: heavy equipment movement, trenching, dirt storage and personnel movement. Air samples collected near Buildings 2 and 3 contained elevated soil fungi levels as compared to outdoor air samples taken in areas unaffected by construction activities. Soil fungi often dominated the samples collected from within depressurized exterior/scaffold containments in Building 3. These findings show that disturbances of soil at active construction sites allows aerosolized *Penicillium* and *Aspergillus* species to be entrained into adjacent depressurized containments (Table 4).

CONCLUSION AND IMPLICATIONS

Soil fungi will often dominate airborne fungal spores at outdoor construction sites undergoing soil disturbance. If negatively pressurized containments are used in areas (e.g., building envelope or scaffold) where soil is aerosolized, soil fungi may dominate clearance air samples. Appropriate interpretation of clearance air sampling should therefore include the collection of outdoor air samples representative of possible construction activities at the site. In some cases, it may be impossible

to achieve a typical (phyloplane) outdoor air rank order of spores in clearance samples within exterior/scaffold depressurized containments.

Clearance criteria for exterior/scaffold containments, therefore, should primarily focus on a thorough visual inspection to assure that all moldy materials and dusts generated during remediation activities have been removed from the containment. Additionally, it should be documented that remediation and clean-up protocol specifications are followed and that appropriate containments have been constructed (AIHA, 2001).

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ASSESSMENT AND REMEDIATION OF MOLD CONTAMINATION IN FAN COIL UNITS OF A 341-SUITE CONDOMINIUM – A CASE STUDY

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ABSTRACT

The overall objective of this project was to provide mold and mechanical solutions for the assessment, remediation and upgrading of 465 mold-contaminated fan coil units in a condominium.

To facilitate the prioritization of the unit-by-unit remediation schedule, an investigation was conducted in each unit including viable air sampling. The results of the air sampling analysis and visual assessment indicated evidence of mold amplification in approximately one-half of the suites. Elevated levels of *Aspergillus versicolor* were identified in many suites.

As a key requirement of the project, the remediation and repair activities were planned and conducted in such a way as to reduce potential mold exposure for occupants and workers and to reduce the potential for further moisture accumulation and mold growth in these units. Preventative measures included upgrading each fan coil unit to allow for better drainage, ease of cleaning and an overflow alarm. A maintenance program and flood response plan was also initiated.

INDEX TERMS: Mold Remediation, Assessment, Prevention, Condominium, Fan Coils

INTRODUCTION

This case study presents the assessment and remediation of mold contaminated fan coil units in a 341-suite condominium. The overall objective of this project was to provide a solution to mold and mechanical issues associated with the fan coil units at the subject site. Throughout the project lifecycle, activities were carried out in such a way as to reduce the potential mold exposure to mold for occupants and workers.

During routine maintenance, mold was observed inside the fan coil units, thus there were concerns about opening and disturbing the interior insulation and systems of the fan coil units. The condominium corporation decided to investigate the extent of the suspected mold problem.

A method of assessing the extent of the mold problem in the fan coil units, while limiting the spread of mold through the suites and minimizing disturbance to the occupants was required. Due to financial and scheduling limitations, all of the fan coil units could not be remediated at once. The criteria for analyzing and prioritizing the order in which the suites were to be remediated were also required.

Some numerical data on interpreting air sampling results is provided by Health Canada (1995). Using data collected from over 3,000 samples in hundreds of buildings, the Canadian Federal-Provincial Advisory Committee on Environmental and Occupational Health in 1995, published interpretation guidelines for microbial measurements in building environments. The guidelines are based on viable air samples collected in mechanically ventilated buildings, using a Reuter Centrifugal Sampler (RCS).

METHODS

The subject building was a 22-storey high residential condominium building constructed around 1990. It consisted of 341 suites, 1 or 2-bedrooms, heated and cooled by a 2-pipe fan coil unit system. The make-up air was supplied from the corridors. Depending on the size of the condominium unit, there were 1 or 2 fan coil units in each suite, all were vertical units furred in drywall enclosures. Air was distributed throughout the suite via ductwork with air grilles on wall or bulkheads. There are also fan coil units serving common areas of the building (i.e. the party room). In total, approximately 465 fan coil units were located throughout the subject building.

The management firm, condominium board of directors, and environmental and mechanical consultants concluded that an effective way to deal with the remediation and upgrading of approximately 465 fan coil units would be to establish criteria to determine priority among the 341 suites. In order to determine the prioritization of the unit-by-unit remediation schedule, an investigation was conducted in each unit. The investigation included the completion of a site survey form, a visual assessment of the exterior of the fan coil unit and enclosure and the collection of a viable air sample.

The rationale for conducting viable air sampling was to aid in the determination that a source of mold was present in the suites and to assess (in a non-intrusive manner) if the identified suspected source (the fan coil units) was impacting the

indoor air. Previous inspections of a sampling of units had indicated the presence of mold inside the fan coil units, thus there were concerns about opening and disturbing the interior insulation and systems of the fan coil units.

Viable air sampling was chosen to allow for a qualitative characterization the samples according to genus and species. The sampling was conducted in very cold weather in December, with snow cover on the ground. We therefore did not expect the outdoor reference samples to identify much, if any, airborne mold. As an alternative to comparing indoor results against outdoor results, the protocol generally chosen, we conducted a qualitative characterization of the samples according to genus and species as part of the qualitative analysis, following Health Canada guidelines (1995). This document cites guideline based on Reuter Centrifugal Sampler (RCS) sampling, therefore, this was the instrument chosen for the project.

One viable mold air sample was collected in each of the suites using a Reuter Centrifugal Sampler (RCS). For every sample collected, the flow rate of the RCS was 40 liters per minute and the sampling time was 4 minutes, for a total sample volume of 160 litres. Rose-Bengal-Agar strips were used for the sampling. The sampling was conducted in general accordance with the procedures described elsewhere (AIHA, 1996).

Air samples were collected directly in front of the supply vent of the fan coil units with the fan turned on. Outdoor samples, make-up air reference samples (from the corridors) and blind duplicate samples were also collected.

At the time of the sampling, a checklist was completed documenting visual observations of suspect mold and/or moisture around the fan coil unit, the dust levels in the suite, the number of plants and whether or not there was evidence of pets in the suite.

Samples were submitted daily for laboratory analysis. The total number of colonies were enumerated by visual examination and a concentration of total fungi were reported in terms of colony forming units per cubic meter (CFU/m³). Colonies were identified by the laboratory according to genus and species and the concentrations of each species of fungi were reported in terms CFU/m³.

RESULTS

The presence of mold was discovered during a routine maintenance inspection growth. While changing the fan coil unit filters, a mechanical contractor observed suspect mold on the cloth covered glass fibre unit cabinet insulation and on the supply air plenums. A number of swab samples were collected and submitted for laboratory analysis and mold growth was confirmed. In addition a number of defi-

iciencies to the fan coil units were noted. It was noted that most of the condensate drain pans were dirty and rusted, condensate tubings were dirty and partially clogged, and the branch pipes were secured on the coil framework with corroded pipe brackets. The coils and fans were found to be in fair condition. These observations were consistent for a number of randomly selected fan coil units inspected.

The results of the investigation, including the air sample analysis, suggested that a mold problem was present in the fan coil units throughout the building. Evidence of mold amplification in was identified on over half of the air samples collected in the suites. Elevated levels of *Aspergillus versicolor* (i.e. over 500 CFU/m³) were identified in many suites.

The assessment results were analyzed and a priority list of suites to be remediated was generated. Priority levels were established through qualitative analysis and using the Health Canada guidelines (1995).

Priority 1: Microbial counts in colony-forming units per cubic meter of air (CFU/m³) exceeded 500 for *Aspergillus versicolor*, or 7 CFU/m³ for *Stachybotrys chartarum*.

Priority 2: Over 150 CFU/m³ of *Aspergillus versicolor*, or over 500 CFU/m³ total fungal count.

Priority 3: Over 7 CFU/m³ of *Aspergillus versicolor*, or over 150 CFU/m³ total fungal count.

Eight suites were designated as Priority 1, 31 suites Priority 2 and 128 suites Priority 3. In addition, 6 units were added to the schedule to due to flooding and 4 units were added at the request of occupants.

The Priority 1 units and any units where health concerns were raised or visible mold was observed in the vicinity of the FCU were remediated and upgraded first. Over the following two months all of the Priority 2 and 3 units were remediated and upgraded at a rate of 5-8 units per day. In addition, the units in which suspect mold or evidence of moisture damage was observed around the fan coil units during the air sampling were also remediated and upgraded. During the 2 month work schedule, if any un-remediated units experienced flooding, they were added to the schedule.

Remediation and Repair of the Fan Coil Units: During the design of the remediation project, the inherent difficulties of remediating mold within a private home was considered.

Mold Remediation: The mold remediation was conducted in general accordance to the NYC Health Department Guidelines (2000). The New York City Guidelines states that a Level V a clean up is defined as a small isolated area of contamination (less than 10 sq ft) in a HVAC system. Since the amount of visible mold below this, Level V procedures were used as a minimum for precautions. Enclosures were erected around the work area, and negative pressure within the containment was maintained during the remediation.

Mechanical upgrade: In order to address the issues of the fan coil units which lead to the formation of mold a mechanical engineering firm designed remedial solution, that included removal and disposal of the existing insulation, cleaning and reuse of the coil and blower.

The original insulation was replaced with a new insulation that consisted of glass fibre insulation with a plastic coating in the mid section of the fan coil unit cabinet where the condensate pan was located and where most of the moisture was expected to be present. The plastic coating was chosen to allow for the easy cleaning of the surface and be less conducive to supporting mold growth, while still providing the sound attenuation required.

Overflow sensors coupled to an indicator light were also installed in the drip pans of the fan coil units. Should the level of water in the drip pan exceed approximately 1/2 inch, the sensor is triggered and the fan will automatically turn off to prevent further condensation. An indicator light next to the wall mounted thermostat will turn on signalling that the unit is overflowing. Instructions were distributed to the occupants indicating that they must contact security immediately if the overflow indicator light turns on.

DISCUSSION/CONCLUSIONS

The presence of mold in the supply vent air sample results did not appear to relate to observations made in the suite, such as the mold on interior surfaces on the suite, the cleanliness of the suite, or the presence of numerous plants. Review of the air sample analysis appeared to indicate that the source of the mold identified was likely from mold amplification inside of the fan coil unit. This was confirmed during the remediation, during which mold was observed on inside of the fan coil units.

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Chapter 11

Prevention and Control

Session chairs:

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BIOLOGICAL ASSESSMENTS FOCUSING ON MOLD

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ABSTRACT

There is an increasing trend in the number of field requests for mold sampling and analysis resulting in a ten-fold increase in mold samples from 2000 to 2002. The development of an assessment procedure for OSHA inspections is a response to worker complaints due to suspected mold exposure and contamination in water-damaged buildings. This approach to assessment utilizes a team-focused inspection process. The team includes a Sample Contracting Coordinator (SCC) who facilitates and assesses mold sampling and analytical requests, well trained field inspectors; and the contract laboratory representatives. Appropriate supplies and equipment; proper chain of custody; and communication between SCC representative, inspectors, and the analytical contract laboratories are essential components of this approach. An inspection includes an onsite evaluation with information gathering via interviews, building assessments, epidemiology modeling, review of medical information, and interpretation of analytical results. A review of this approach to biological assessments used at the OSHA Salt Lake Technical Center (SLTC) has shown the importance of providing assistance to field personnel, including publication of recommendations for conducting building inspections, improved inspection tracking and support to keep up with the increase in customer requests, and distribution of guidelines on appropriate building repair and maintenance. The challenge is to continually evaluate the requests for assistance and make recommendations for improvements to existing procedures for better customer service to Compliance Officers within budgetary constraints.

INDEX TERMS: Mold assessment

INTRODUCTION

Public awareness and concern regarding indoor exposure to mold has increased, in part, due to information from both scientific publications and the mass media. This awareness and concern has educated and informed employees in indoor occu-

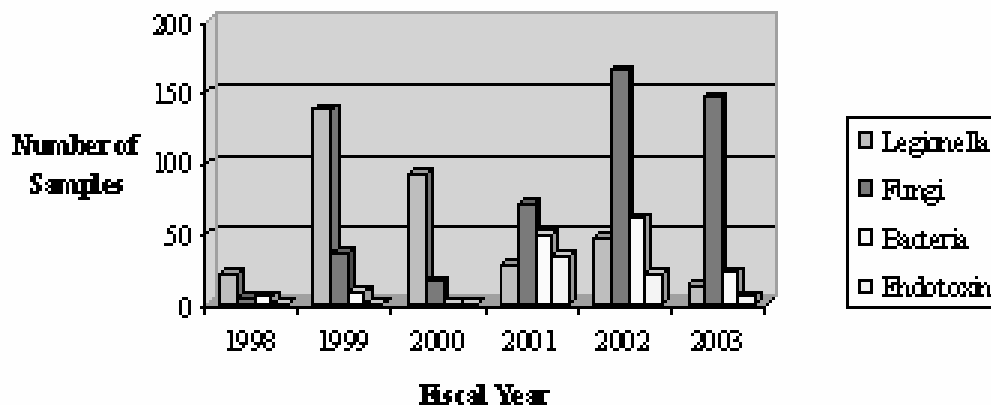
pational settings regarding mold exposures and the possibility that these exposures may cause health symptoms. Several publications and government documents recognize mold as a possible health hazard and recommend that if visible mold contamination is present it should be removed and re-occurrence prevented. Employer compliance with hazard-specific safety and health standards is required by the Occupational Safety and Health Act and is enforced by either Federal Occupational Safety and Health Administration (OSHA) or an OSHA-approved State Plan. Currently, there is no specific OSHA standard for mold exposure. In order to support OSHA Compliance Officers in mold inspections, the Salt Lake Technical Center (SLTC), Industrial Hygiene Chemistry Division (IHC) has developed a procedure to support field requests for sampling and analysis.

DISCUSSION

Evaluating the Number of Mold Samples for Trends

The number of biological samples collected and analyzed by OSHA for each fiscal year (FY) from 1998 through March 2003 was evaluated. Mold sample numbers were totaled and compared to sample totals of three other biological categories, consisting of *Legionella*, Bacteria, and Endotoxin. In comparison, we found that *Legionella* samples predominated for FY 1998 through FY 2000 with a decrease from FY 1999 to FY 2001. There were 4 mold samples in fiscal year 1998, and 36, 16, 70, 166 for FY 1999, 2000, 2001, 2002 respectively. For FY 2003, the data is limited to six months with 147 mold samples. The trend is a significant increase of mold samples for all years except 2000. The increase from FY 2000 to FY 2002 was approximately ten fold. (Figure 1)

Figure 1. Biological samples, FY 1998 – March 2003



IHC supports OSHA field personnel in mold inspections by facilitating sampling and analytical requests and has developed an internal Standard Operating Procedure (SOP) to ensure that contracted samples are processed in a manner consistent with AIHA Laboratory accreditation procedures.

Standard Operating Procedure for mold sampling and analysis requests:

The SOP used by OSHA:

Identifies which IHC laboratory staff to function as Sample Contracting Coordinators (SCC) whose responsibility is to facilitate sampling and analysis requests by completing assessment and intake of each request.

Describes procedures for authorization of sampling before samples are collected.

Suggests a review of sampling plans by the SCC and the Compliance Officer and makes recommendations, where appropriate, to assure efficient sampling is performed and includes the Assessment Process of the Onsite Inspection.

Requires forwarding to the OSHA Area Office, contract laboratory information (Appendix A).

Requires submitting to the contract laboratory, authorization to analyze a specific number and type of samples as well as OSHA Area Office information (Appendix B).

Ensures that the Compliance Officer is instructed to collect samples on media recommended by the contract laboratory.

Identifies steps to ensure quality sample contracting and tracking, including computer sample log in, assigning of official sample numbers, forwarding sample numbers to contract lab, official OSHA form processing, chain of custody, and checking of sample integrity.

Requires completion of an inspection tracking form for each request for collection and analysis of samples using a contract laboratory.

Identifies procedures for selecting a contract laboratory, forwarding sample results, entering of results into the SLTC database, releasing of results and review by SCC, and methods of reporting of results to OSHA Area Office.

Identifies requirements for contract laboratories that qualify them to receive samples.

This IHC procedure provides customer service support by a designated SCC to the OSHA Compliance Officer. The role of the SCC is to facilitate the mold sampling request and provide technical support to the Compliance Officer. The process

begins with the SCC being contacted by the Compliance Officer who has been assigned a biological inspection. The SCC facilitates the request for sampling and analysis by an initial review and assessment of information provided by the compliance officer. This information will include identifying the reason for the complaint, the number of employees at the site, the number of employees with symptoms, the description of symptoms, the medical documentation for symptoms, the type of building, the type of work the company is involved in and the work assignments of the employees, the water history of building, geographic information (e.g. high water table), the presence of visible signs of mold, etc. The procedure continues with the SCC providing technical assistance and evaluating pre and post inspection status updates. If several employees are experiencing symptoms consistent with mold exposure, if there is a clustering of employees, medical documentation, etc., the SCC will coordinate mold sampling and analysis for the Compliance Officer. The SCC then provides supplies and information for sampling, selects and coordinates with a specialized contract laboratory for the analysis, ensures the chain of custody for samples is in place, fills out tracking forms and forwards them to the Compliance Officer and contract laboratory, ensures IHC management and other SCCs are informed of the inspection, arranges for analytical interpretation by specialist if needed, and reviews and provides analytical results to the Compliance Officer.

Assessment process of the onsite inspection: The assessment process relies on information from the onsite inspection. The SCC provides support to the Compliance Officer by providing assistance which can vary depending on the type of support requested. The decision to inspect and the performance of the inspection is the responsibility of the Compliance Officer and as such the SCC position is only an advisory and supportive role. However, those Compliance Officers requesting additional assistance are provided with more detailed inspection recommendations by the SCC.

Prior to going onsite, it is recommended that the Compliance Officer plans and gathers preliminary information as well as organizes supplies and equipment. An onsite assessment includes a continuation of information gathering similar to SCC intake questions. Management, maintenance representatives, and occupants are interviewed for the history and use of the building. The water damage history of the building as well as repairs, construction and materials are reviewed. The building records are evaluated and assessed for any water damage events with duration, dates, and outcomes. Other inspection components are assessed and include a review of the building's occupancy and use; identifying employees adverse health symptoms and any medical documentation; assessing the possibility of symptoms being building related; describing the building (number of floors, total area square feet, construction, etc); and gathering information of occupants (number of occu-

pants, locations of different departments and work tasks). The number of employees with symptoms and location within the building is evaluated as well as any clustering of employees with symptoms. Medical information on employees with symptoms is acquired. An inspection of the site exterior for water damage and incursion is completed.

An inspection of interior spaces is performed for water and moisture. Temperature, carbon dioxide levels, and humidity are determined. Heating, ventilation, and air-conditioning (HVAC) systems and other equipment and occupied spaces are evaluated. In addition, evaluations are performed with regard to moisture, potential sources of mold, and possible pathways and disseminations that could cause employees symptoms (source-pathway-exposure).

A theory (hypothesis) using epidemiology evaluation of person, place and time to determine possible causes and linkage to employees with symptoms may be developed. The theory (hypothesis) is tested and a determination if sampling is necessary is made. (Note if visible signs of mold are present, samples are not usually taken as part of the inspection, but may be in order as part of the remediation process. It is recommended to remove the mold and prevent re-occurrence). If sampling is necessary (e.g. confirmation of reservoir) the SCC is contacted for facilitation of sampling and analysis. Analytical results with all other information including employee medical information are interpreted and evaluated. A mold specialist may be needed to assist in the analytical interpretation. The need for remediation and recommendations are evaluated by the Compliance Officer. Some Compliance Officer's request additional information on remediation and the Compliance Officer is provided with this information in the form of references as part of the customer service program (e.g. EPA Documents on building maintenance and remediation).

Below is a case study example of an OSHA mold inspection in which SCC recommendations and technical support added value to the inspection and the outcome.

CASE STUDY

Background: The Compliance Officer contacted the SCC regarding a complaint of mold issues by employees of company A in a single level strip mall. The Compliance Officer had little experience with biological inspections and requested technical support regarding the onsite inspection process. The SCC provided inspection recommendations and discussed intake questions. The Compliance Officer went on site and reported to the SCC of several employees with symptoms, including headache, nasal and eye irritation, fatigue and breathing problems. The Compliance Officer could not see any visible signs of mold and, in addition, there was no history of water damage. The SCC noted that employees with symptoms

were clustered in one location of the company and recommended that the Compliance Officer revisit this location and re-evaluate the area not just visually, but also by touching each wall, the ceiling, floor, etc.

Finding: The Compliance Officer located one wall that was damp to the touch. This wall was the only wall that was shared with another company, B situated adjacent to company A in the same strip mall. The Compliance Officer requested an interview with the manager and employees of company B and was invited onsite. Visible signs of mold were observed in company B due to water damage from plumbing problems with a leaking sink attached on the shared wall between the two companies.

Remediation: Company B fixed the plumbing leak and replaced a majority of the shared wall. Company A employee symptoms began to resolve. By following the recommendations by the SCC, the inspector was able to broaden the parameters of his search and determine the true source of the mold.

CONCLUSION

Mold contamination in occupational settings can be harmful to employees. OSHA mold inspections are a response to worker complaints and referrals from targeted inspections. Adverse health effects due to suspected mold exposure and contamination are often part of the complaint. An effective process of providing technical support to investigators who evaluate mold complaints in occupational settings can be an effective asset in resolving the causes of those complaints. The described process for performing biological assessments concerning mold has been in use in the Industrial Hygiene Chemistry Division of the OSHA Salt Lake Technical Center for approximately two years and has proven to be very successful in meeting OSHA field staff needs.

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IMPACT OF VENTILATION DESIGN ON FUNGAL PROLIFERATION IN A SCHOOL HVAC SYSTEM

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INTRODUCTION

Appropriate ventilation system design and operation are key factors for minimizing conditions that may promote fungal growth in indoor environments, such as schools. An indoor environmental quality (IEQ) investigation was conducted at a two-story New England grammar school (the School) in September 2002 in response to reports of musty odors and stained ceiling tiles in multiple interior rooms located on the first and second floors. This investigation followed an extended spell of hot, humid weather in August 2002 when air-conditioning was in use at the School.

A comprehensive IEQ investigation was designed to identify potential sources of moisture accumulation and fungal growth as likely causes for the musty odors and stained ceiling tiles at the School. Visual inspections in classrooms and offices and in accessible areas of ventilation ductwork were completed. A comprehensive sampling plan for fungal materials that included collection of air, surface, and bulk samples from interior rooms and perimeter rooms at the School was also designed.

METHODS

Air samples for analyses of airborne levels of viable and non-viable fungal spores were collected with a Burkard Personal Volumetric Spore Sampler (Burkard Manufacturing Co., Hertfordshire, England). Samples were collected onto coated glass slides for five minutes. Surface samples were collected for analyses of the presence of fungal materials by applying clear adhesive tape to a selected surface and then transferring each tape to a glass slide. Bulk samples of various building materials were also collected for analyses of the presence of fungal materials. All samples were sent to Environmental Microbiology Laboratory (San Bruno, California) for analysis by light microscopy.

RESULTS

The basic design for the School's heating, ventilation, and air-conditioning (HVAC) system included the use of unit ventilators without cooling or dehumidifying capability in perimeter rooms, which were defined as rooms with at least one outside wall. Fan coil units with cooling capability served the interior rooms. The ductwork serving the interior, air-conditioned rooms was internally lined with fiberglass insulation.

During the initial inspection of the School, bowed ceiling tiles as well as water stains and wet ceiling tiles beneath the fan coil units in the interior rooms were noted. Many interior rooms felt overcooled. Due to this overcooling, many school staff members had propped open their doors to control temperature. The subsequent conclusion was that this practice facilitated the migration of warm, humid air from the perimeter rooms and hallways that lacked air-conditioning toward the interior, air-conditioned rooms. The result was condensation of the moisture-laden air on the cooled surfaces in the interior rooms and subsequent mold growth on a variety of wetted building materials.

Results of air samples collected from representative interior rooms during the initial inspections are presented in Table 1. Total spore count values include all spore types in each air sample.

Table 1. Results of analyses for viable and non-viable fungal spores in air samples collected with a Burkard personal volumetric sampler at the school

Location	Penicillium/ Aspergillus (spores/m ³)	Cladosporium (spores/m ³)	Basidio-spores (spores/m ³)	Total (spores/m ³)
Interior Room 100	4,150	370	444	5,112
Interior Room 107	9,930	815	74	10,987
Interior Room 118	2,820	296	222	3,450
Interior Room 120	519	741	222	1,575
Interior Room 121	8,740	370	74	9,222
Interior Room 205	667	519	963	2,242
Main Lobby	556	19	111	742
Outdoors	2,370	8,150	4,590	16,483

The air sampling results indicated elevated levels of *Penicillium/ Aspergillus* spores in several interior, air-conditioned rooms that were 2 to 4 times higher than the outdoor level of 2,370 spores per cubic meter of air. These results indicated indoor sources for the growth of this fungal genera.

Results of surface and bulk samples collected from representative interior rooms during the initial inspections are presented in Table 2.

Table 2. Results of analyses for fungal materials in bulk and surface samples collected at the school

Location	Sample Description	Molds with Mycelial and/or Sporulating Structures 1	Impression
Interior Room 113	Surface Sample – Above plenum on duct	3+ <i>Cladosporium</i> 1+ colorless spores typical of <i>Penicillium/ Aspergillus</i>	Mold growth
Interior Room 113	Surface Sample – Staining on tile at diffuser	3+ <i>Cladosporium</i>	Mold growth
Interior Room 113	Bulk Sample – Duct lining at diffuser	4+ <i>Cladosporium</i>	Mold growth
Interior Room 107	Surface Sample – Discolored area of pipe insulation	4+ <i>Cladosporium</i>	Mold growth
Interior Room 107	Surface Sample – Supply diffuser grille	3+ <i>Cladosporium</i>	Mold growth
Interior Room 121	Surface Sample – Stained pipe insulation	3+ <i>Cladosporium</i>	Mold growth
Interior Room 118	Surface Sample – Supply diffuser grille	4+ <i>Cladosporium</i>	Mold growth

¹ Quantities of molds seen are graded 1+ to 4+, with 4+ denoting the highest numbers.

The results indicated moderate to high (3+ and 4+) levels of *Cladosporium* in a majority of the samples, particularly those from HVAC components. These results

indicated indoor growth of this fungal genera on HVAC components and other surfaces.

Additional inspections were conducted to determine the presence and extent of mold growth in the ductwork of the interior rooms. Surface and bulk samples were collected from areas within the ductwork on the first and second floors of the School. The sample results indicated that the majority of ductwork serving the interior air-conditioned rooms exhibited some level of mold growth on the interior surfaces.

Based on these sample results, the recommendations were either to remove or encapsulate the internally-lined ductwork in all interior rooms; the School administration elected to encapsulate the ductwork. The encapsulation process required vacuuming of the internal fiberglass insulation prior to application of a sealant material to the inside surfaces of the ductwork in all interior rooms.

The procedures utilized were in accordance with the guidelines provided by the United States Environmental Protection Agency (2001) and the New York City Department of Health (2002) to investigate and remediate the mold growth in classrooms and offices. The remediation efforts included construction of full polyethylene containment structures around all work area and maintenance of negative pressurization in these work areas relative to adjacent spaces. Clearance testing procedures were used to verify the effectiveness of the remediation efforts at each location in the School. In addition, each containment zone met the established clearance criteria before they were categorized as clean and then disassembled. The clearance criteria included the following:

- No evidence of mold growth, water staining, or mold or mildew-like odors.
- No evidence of visible dust or debris.
- Collection of air samples for fungal spores from below the remediated ductwork while the HVAC system is operating. Results from these air samples would be interpreted relative to results from outdoor air samples to determine acceptable indoor spore levels.
- Re-cleaning and re-evaluation of the ductwork are repeated until the clearance criteria are met for each tested area.

DISCUSSION

Remediation of fungal growth problems caused by the condensation of moisture-laden air on the overcooled surfaces in the interior rooms resulted in a costly project for the school administration. It was later learned that the initial HVAC design for the school included air-conditioning for the entire building. Implementation of

this initial design plan or a choice not to air-condition the School at all would have precluded the water condensation and subsequent fungal growth problems that occurred in the interior core rooms of the School during the August 2002 hot spell. It was recommended that the School administration adopt a proactive program that includes administrative controls to minimize transfer pathways for moisture-laden air into the air-conditioned areas. A monitoring program for moisture conditions that could result in mold growth at the School was also recommended.

CONCLUSIONS AND IMPLICATIONS

This investigation demonstrated the importance of careful consideration of the potential impact of ventilation design on conditions that may promote fungal proliferation in indoor environments, such as schools.

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MOLD IDENTIFICATION AND CONTROL STRATEGIES IN A CANADIAN TRAINING COMPLEX

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ABSTRACT

This paper describes building-related mold contamination issues in a large training centre from 1994 to 2002. Five full-scale mold assessments were performed and several physical control strategies were implemented to reduce mold growth and the potential for spore spread through the buildings. Recent annual follow-up assessments have seen a decrease in contamination in the mechanical systems and the crawlspaces below the building. This paper explains the physical control strategies used over the period of study and attempts to reconcile their implementation with the observed mold spore counts. Data collected indoors, outdoors, and in the crawlspaces over the length of the study is presented and discussed.

INDEX TERMS: Mold Remediation, Sampling, Physical Control Strategies

INTRODUCTION

The centre was built as a senior high school and has been mainly used for language training since it was acquired by the current owner. The centre consists of 26 linked one- and two-storey blocks situated in a lush surrounding. The blocks house either classrooms or offices, and also common utilities such as washrooms, reading areas and canteens. The building design features exposed wood beams and large windows and the configuration of the roof does not allow for HVAC components to be installed on it. Each block has its own ventilation system housed in an unpaved crawlspace at basement level. Most blocks have two air handling units, each with a separate air intake and exhaust. Most of the air intakes were initially located only 0.3-1 m above ground level. Incoming air underwent heat exchange with crawlspace air that was being exhausted, and then was directed to heat pumps where it was mixed with return air and distributed to occupied areas.

During a routine health and safety inspection in 1994, Occupational Health and Safety (OHS) officers refused the facilities management personnel access to the

mechanical systems located in the crawlspaces of the centre. Their concerns included safety in the confined space and exposure of maintenance personnel to what appeared to be mold. Persistent health and musty odor complaints from occupants also pointed to potential mold contamination in occupied areas. Evaluation of the exact environmental conditions and corrective action was required by OHS immediately, starting the series of major studies described below.

METHODS

October 1994: The first investigation performed in October 1994 involved a full-scale Indoor Air Quality (IAQ) investigation and a mold sampling survey.

Indoor Air Quality Study: The IAQ investigation consisted of a walkthrough of all blocks and crawlspaces to identify IAQ problem indicators and contaminant sources. Measurements of temperature, relative humidity (RH), carbon dioxide (CO₂), carbon monoxide (CO), total volatile organic compounds (TVOC) and respirable suspended particulates (RSP) were taken with direct reading equipment in 345 rooms of the centre. Limited sampling for formaldehyde and radon gas was also performed.

These indicators of indoor air quality, with the exception of the temperatures in some rooms, were all found to be within accepted ranges for classrooms and offices. The crawlspace inspection found dampness and mold problems and recommended engineering rather than chemical solutions.

Mold Sampling: The methodology used in the 1994 and all subsequent studies consisted of airborne culturable microbial samples taken with a Reuter Centrifugal Sampler (RCS). The RCS employed rose Bengal agar as a medium to enhance mold and yeast capture and identification. Before and after the collection of each sample the head and impellers of the RCS were disinfected to avoid contamination between samples. Care was taken to seal used agar strips properly in their original package, to provide refrigerated storage, and to ensure sample delivery to the laboratory within 24 hours.

In 1994, a total of 134 samples were taken over a 3 day period to allow for comparison. The majority of the samples (69) were taken in occupied spaces, with the remainder performed in crawlspace areas (38), air intake pits (21) and outdoors (6).

The analyses were performed by laboratories using established methods published by the American Industrial Hygiene Association. The laboratories conducted a 3-week culturation period and analyzed the samples to determine the overall number of colony-forming units present at the sampling point (CFU/m³ air) and to give a speciation of the molds and fungi present in the sample.

The results and conclusions of the mold sampling were:

1. Contamination from soil and organic debris was inevitable with the intake design.
2. The air in the crawlspace was unacceptable for worker exposure without a face mask. The fungal spore loads were largely in excess of those associated with organic dust toxic syndrome and hypersensitivity pneumonitis.
3. Some vents were found to suck crawlspace air into the HVAC system. There was a potential for crawlspace air to enter all systems through leaky return air ductwork.
4. Crawlspace accessibility was a problem.
5. The crawlspace inspection revealed that the dampness was not caused by water intrusion through foundation walls but was intrinsic to the dirt floor. A follow-up engineering study found that the dampness was due to a high water table and poor drainage.
6. Some contamination was found in occupied spaces along with persistent complaints from building occupants regarding musty odors and health problems.
7. All outdoor mold spore samples were rated Too Numerous To Count (TNTC), or in excess of 3000 CFU/m³ by the laboratory.
8. The cafeteria was found to be contaminated with toxigenic species of mold.

Corrective Actions: The following improvements were carried out to the centre:

1. Outdoor air intake raised to roof level (3 m above ground) in two blocks.
2. Replacement of soil and crushed stone with concrete in the air intake pits.
3. Sealing of ductwork located in crawlspaces.
4. Clean-up of several blocks using high efficiency particulate air (HEPA) vacuum cleaners.
5. Cleaning of wall panels in several classrooms where a yellow substance associated with mold was observed.
6. Installation of a new ventilation and cooling system in the cafeteria.
7. Maintenance personnel were recommended to wear face masks on health grounds when working in the crawlspace.

August 1995: As a follow-up, another extensive mold and IAQ study was performed in August 1995. The results are summarized below:

1. The level of dampness in the crawlspaces appeared to be slightly lower than what was found previously.
2. Far fewer intakes showed the presence of soil fungi. This was probably due to the improvements made to the intake pits.

3. The intakes which had been raised to roof level showed much lower spore counts.
4. Mold spores were in the “normal range” for most occupied rooms. Some rooms were flagged as contaminated.
5. All outdoor mold spore samples were TNTC.

Corrective Actions: All blocks which had been labeled problem areas were fully cleaned using high efficiency particulate air (HEPA) vacuum cleaners and surface wiping.

November 1995: Further IAQ and mold investigations revealed that:

1. The cleaned blocks were found to have low spore counts.
2. The level of dampness in crawlspaces was similar to previous studies.
3. Grit, dirt and dust (still) present “on the new surface of some of the intakes” and “in front of most of the intake dampers”.
4. Outdoor mold spore counts were found to be very low. The outdoor temperature was 0 °C on the day of testing.

Corrective Actions: A new drainage system was installed around the centre to improve drainage around the building foundation and dry out the affected crawlspaces.

1. Access to the crawlspaces was improved by excavation to increase crawlspace height and construction of concreted floors in drier areas.
2. All outdoor air intakes were raised from approximately 1 m to 3 m above ground.
3. New air-handling units with better filters replaced more than half of the 30-year-old units. The heat pump filters were removed on these units, and the filter change out slots sealed off to prevent crawlspace air entry into supply air.
4. All ductwork under negative pressure in the crawlspaces was replaced with high pressure ductwork to prevent air infiltration from the crawlspace. All new ductwork was tested with a fog machine to pressure test all the joints prior to connection to the system. The new ductwork was installed without any acoustical liner to facilitate cleaning and prevent mold growth within the ductwork.
5. New exhaust fans with pressure sensors were installed throughout the crawlspace to maintain the crawlspace under negative pressure at all times relative to occupied areas. The fans exhausted directly to the outdoors and also acted to lower the humidity level in the crawlspace.
6. Gaps in floor slabs in some blocks which had been allowing crawlspace air to enter occupied spaces directly were sealed.

JUNE 1997: Following completion of the above corrective actions, mold and IAQ study conducted in June 1997 revealed the following:

1. The crawlspaces appeared drier and had significantly lower mold spore counts.
2. The cement bases of crawlspace intake pits were found to be cleaner. The improved access to the crawlspaces and intake pits enabled them to be properly cleaned.
3. Toxigenic molds were identified in five of the samples taken in occupied spaces, three of which appeared to have a history of problems. Cleaning hard surfaces with bleach and vacuuming with HEPA vacuum was recommended.
4. Most intakes had similar spore counts and species to outdoor air.

1998-2002: InAIR Environmental Ltd. performed annual follow up surveys between 1998 and 2002. These consisted of IAQ testing in occupied areas and mold sampling in occupied spaces, crawlspaces, intakes and outdoors. In general, the occupied spaces tested clean with low spore counts and no more than 1-2 spores of toxigenic species found in no more than 3 rooms each year. The spore counts in the crawlspaces remained low and minimal (and often no) spores of known toxigenic species were detected each year. This is a marked change from the 1994-1997 studies when the presence of toxigenic spores was much more common in crawlspace samples.

The data collected is shown in Figures 1 and 2. Figure 1 shows the average spore counts in crawlspaces and outdoors for the study period. In Figure 2, the data has been processed to yield probabilities for measuring spore counts in excess 100 CFU/m³ for the crawlspace of the entire complex. The outdoor spore counts are provided as reference. Though averaging causes some loss of resolution, it allows for comparison of data over the whole study period.

Figure 1. Comparison of average spore counts in crawlspaces and outdoors over the period of study

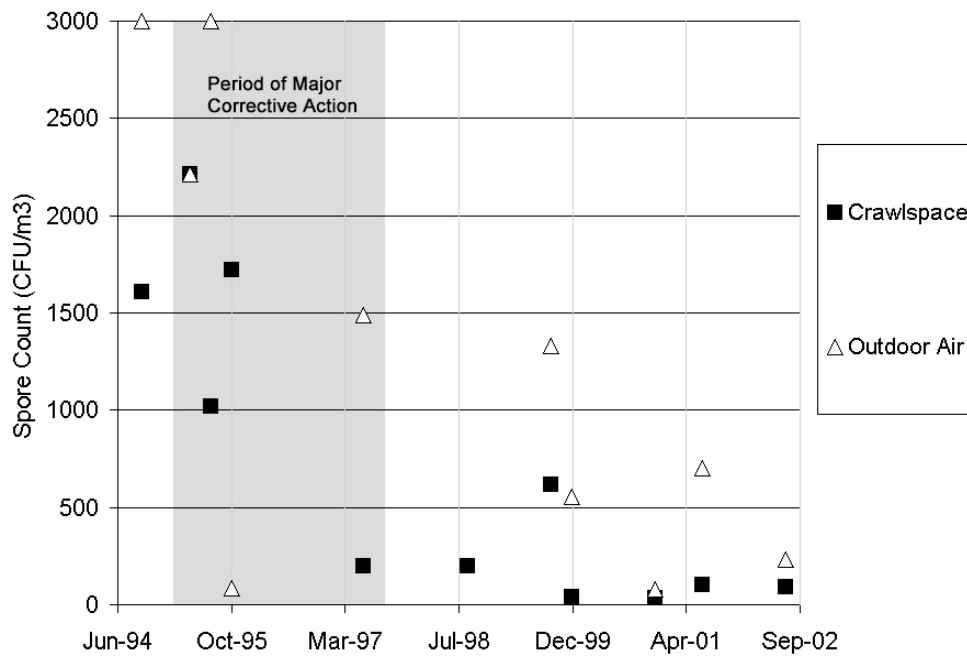
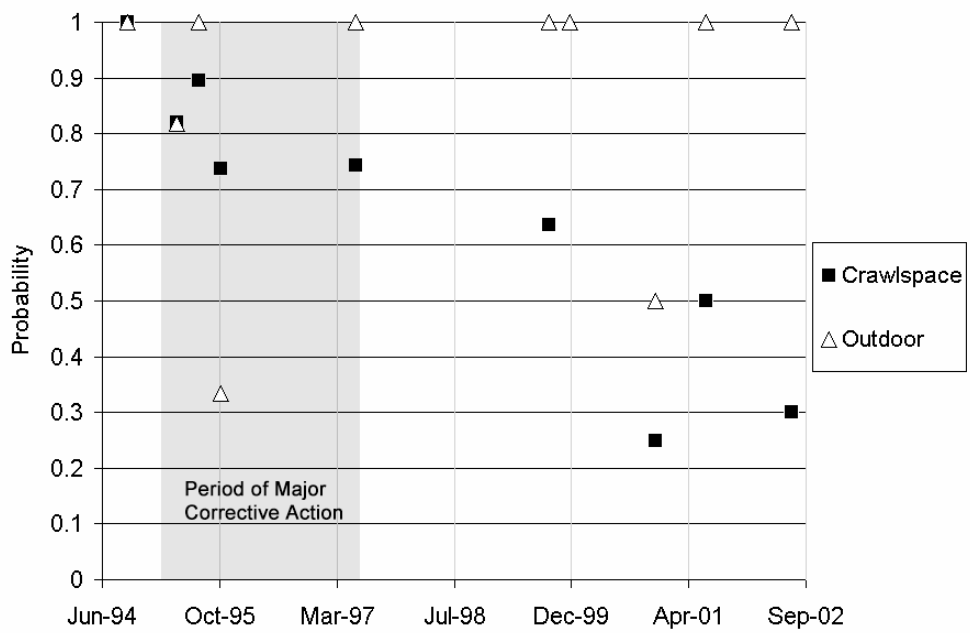


Figure 2. Probability of measuring a spore count in excess of 100 CFU/m³



DISCUSSION

Generally, a significant reduction in numbers of mold spores in the crawlspace occurred over the study period (Figures 1 & 2). Much of the decrease happened during the period of major corrective actions (Figure 1). This period also coincided with a decrease in the presence of toxigenic species in the crawlspaces. These decreases are unlikely to be related to the decrease in outdoor mold spores (Figure 1) because the mycoflora are different in the two cases.

CONCLUSIONS AND IMPLICATIONS

Building design can have a major impact on the potential for mold growth. While physical control strategies may be effective in reducing moisture and addressing building design flaws in older buildings, the importance of proper design and construction can not be overstated. In this case, the potential for mold problems could have been reduced by conducting geotechnical work to improve the site drainage prior to building construction, locating the AHUs at roof level rather than in the crawlspaces, and ensuring the air intakes were located high enough to not entrain soil and organic debris.

RISK ASSESSMENT OF FUNGAL BIOAEROSOLS IN INDOOR ENVIRONMENTS: CURRENT PARADIGMS AND EVOLVING CONCEPTS

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ABSTRACT

Occupational and environmental health professionals are faced with the daunting task of assessing the potential health risk of occupants exposed to fungal-contaminated indoor environments. The potential consequential health effects include: irritation, allergic reactions, infection, and possibly intoxications, depending on the particular fungal species and strain, prevailing environmental conditions, amount and duration of exposure, and the susceptibility of exposed individuals. Toxicological risk assessments are particularly difficult due the fact that many contaminants do not reach acute toxicological levels, but the effects of chronic exposures remain uncertain. Risk assessments are further complicated by the fact that exposures often entail complex mixtures of contaminants. Here we will discuss general concepts, applications and limitations of exposure and qualitative risk assessment. In addition, we will discuss some new or evolving concepts that may aid in assessing the potential health consequences of exposure to bioaerosol contaminants in indoor environments.

INDEX TERMS: Fungal bioaerosols, Qualitative risk assessment, Indoor environments

INTRODUCTION

Exposure to saprophytic fungi growing in wet indoor environments has been linked to a number of adverse health effects including, but not limited to, lethargy, allergies, asthma, dermatitis, hypersensitive pneumonitis and infection (Kordula *et al.* 2002; Dales, Miller 2001). While the aforementioned symptoms are intuitive and largely recognized by both the scientific and lay communities, there are significant secondary health effects associated with exposure to fungi, including idiopathic pulmonary hemorrhage in infants (Etzel *et al.* 1998) cognitive impairment,

encephalopathies (Gordon *et al.* 1993), immuno-suppression (Johanning *et al.* 1996), and cancer (Hayes *et al.* 1984; Wray, 1975). Some researchers have proposed that these latter health effects may be attributed to the mycotoxins produced by the fungi, thus constituting a unique set of agents for health effects reported by victims of fungal exposure. Although the mycotoxin model remains highly controversial, alternative explanations have not been proposed until the recent discovery of hemolysins (Van Emon *et al.* 2003; Vesper *et al.* 1999, 2000, 2001, 2002) and serum proteases from *Stachybotrys* (Kordula *et al.* 2002). Risk assessments following exposure to bioaerosols should factor in all the possible mechanisms, i.e., inflammation from, exposure to β -D-glucans, bacteria and bacterial endotoxins, irritation from mVOCs, allergic reactions, infections, hemolytic activity, and possible toxic responses to mycotoxins, as they relate to the specific circumstance. Attempts to quantify inhalation exposure to molds indoors has been based largely on the assumption that the exposure vehicle for allergy and intoxication is known, that is, that conidia (spores) are the carriers of toxins and allergens. Recent work (Englehart *et al.* 2002; Jacob *et al.* 2002) has shown hyphal fragments, and dust from substrates on which mold grows can contain allergens and toxins. Exposure analyses based on spore counts or viable colony counts likely underestimate exposure to allergens and toxins. Strictures of sampling for spores or viable organisms are due to sampling devices and sampling strategies and on the behavior of molds, and air currents in buildings. These cannot accurately characterize even exposure to spores. Unfortunately, many bioaerosol components lack analytical methods that are sensitive-enough to detect the levels obtained in most contaminated indoor environments. In addition, contaminated environments typically contain mixtures of bioaerosol and abiotic factors with concomitant interactions that may affect occupant health. As such, risk assessments have been reduced to qualitative rather than quantitative models. Here we will review some of the current paradigms and some evolving concepts that may be useful in qualitative risk analyses of bioaerosol-contaminated indoor environments.

METHODS

We conducted internet literature searches on MEDLINE and Medscape (<http://www.medscape.com/>), ASM Journals (<http://www.asm.org>), Ingenta (<http://www.ingenta.com>), and Inforetrieve (<http://www.inforetrieve.com>). Search terms using and/or connections included: bioaerosol, mold, fung*, mycotoxin*, "indoor air", "indoor environments", and "health effects". The literature searches are done monthly to update the database.

RESULTS AND DISCUSSION

Epidemiological Models: Over 25 case and cross-sectional studies are currently in the literature, involving over 45,000 exposed individuals (Fung, Hughson, 2003). In general, the most common health effects were associations with increased incidences of asthma, rhinitis, and upper respiratory complaints. This supports the hypothesis forwarded by many experts, that the most common health effect is probably allergy. Other symptoms that were reported in a few of these studies, include; idiopathic pulmonary hemorrhage and hemosiderosis in infants, interstitial lung disease, and neurological and cognitive symptoms, and effects on the immune system, generally measured as increased rates and duration of viral and bacterial infections.

Of the symptoms associated with mold exposure, infantile idiopathic pulmonary hemosiderosis remains one of the most controversial. Along with the well-known ten cases in the 1994 Cleveland incident (Etzet *et al.* 1998; Fung, Hughson, 2003), associations of hemosiderosis to mold exposure have been described by 27 additional cases in Cleveland (Dearborn *et al.* 1999), Elidemir *et al.* (1999), Flappan *et al.* (1999), Novotny and Dixit (2000) and Weiss and Chideckel (2002). Exposure to second-hand smoke was also associated with the onset of pulmonary hemorrhaging in the infants, suggesting the possibility that the secondary assault from the smoke may have acted synergistically with mold-associated factors and possibly other environmental factors to induce hemorrhaging. Although mycotoxins, especially trichothecenes, were originally suggested as the most-likely causal agent, the recent work of Vesper's group suggests that hemolysins may also play a role in the hemorrhagic onset (van Emon *et al.* 2003).

Physiological Mode of Action:

Allergy: The general consensus appears to indicate that allergy is the most common response to fungal exposure in contaminated indoor environments (Dziadzio, Bush, 2001;). Allergic reactions can range from mild, transitory responses, to severe, chronic illnesses. The Institute of Medicine (1993) estimates that one in five Americans suffers from allergic rhinitis (Type I response), the single most common chronic disease experienced by humans. A much smaller number, less than one percent, suffer serious chronic diseases such as allergic bronchopulmonary aspergillosis (ABPA) or hypersensitivity pneumonitis (Type III response) (Institute of Medicine, 1993). However, the proportion of individuals reporting allergenic-type responses following fungal exposure exceeds the expected population (20%) of atopic individuals. In addition, there is a preponderance of reports of flu-like symptoms suggestive of Type III rather than Type I allergic responses. These could be the early-stages or sub-acute stages of hypersensitive pneumonitis.

Mycotoxins: The study of mycotoxins in indoor environments is relatively new. Dose-response data are largely unavailable due to limitations of sampling putative carriers (spores, fragments, dust), as well as failure to measure toxins in samples during investigations where toxins are found to play a likely role. Difficulties in determining toxins and their metabolites, or biomarkers in human tissues from low-level exposures have also played a role in the current lack of dose-response information. However, theoretical extrapolations have been done using; 1) the health effects that have been observed in agricultural situations, 2) the known biology and biochemistry of the organisms and their specific toxins, and 3) knowledge of toxins from the same classes of compounds. Trichothecenes, for example, are potent inhibitors of protein and nucleic acid synthesis (Buck, Cote, 1991). At low concentrations, they are known to suppress the immune system, making animals (or humans) more susceptible to infection (Buck, Cote, 1991), which may account for the complaints from people in fungal-infested buildings of increased incidences of colds, flu, and nasal congestion (Johanning *et al.* 1996) and the finding in numerous epidemiological studies of increased incidence of viral or bacterial infections. T-2 toxin, for instance, requires 50 to 100 times less toxin to suppress mammalian T and B cell proliferation (a commonly used test for immunosuppression) than to cause cytotoxicity (Miller *et al.* 1997). Infants with immature immune systems and individuals with compromised immune systems would be considerably more vulnerable. As indicated previously, other fungi such as *Aspergillus* and *Penicillium*, which are commonly found in indoor environments, can produce mycotoxins including aflatoxins, ochratoxin, and patulin which are not only extremely toxic but are also immunotoxins.

Complicating the issue is the fact that different strains of the same fungal species (i.e., *Stachybotrys*) produce very potent and less potent toxins. in the field, making it impossible to predict mycotoxin levels based solely on spore concentrations in air (Jarvis, Hinkley, 1999; Jarvis *et al.* 1998). In addition interactions probably occur between the differing mycotoxins, endotoxins, and immunosuppressive compounds that are produced by the microflora as well as the abiotic factors such as VOCs in the contaminated indoor environment. Sensitivity to any single or combinations of these factors can vary orders of magnitude between differing individuals. For instance, the concentration of endotoxin exposure to induce symptoms is over a two-log difference from the most sensitive to most insensitive individual (Milton *et al.*). Both *Aspergilli* and *Penicillia* produce potent neurotoxins, while aflatoxins and their precursors (i.e. sterigmatocystin) and trichothecenes produced by *Stachybotrys*, *Trichoderma* and *Fusarium* and other genera directly affect the brain.

Unfortunately most of the evidence associating mold-exposure to pulmonary hemorrhage and neurological effects are based on epidemiological studies, which do not, by design prove causalities. Only a sufficiently deep body of literature on

specific endpoints can satisfy the criteria for causality. To date no prospective study has been designed to determine mycotoxin roles in illness from indoor exposures and no convincing alternative explanation has been offered, nor compelling evidence provided to refute the hypothesis that mycotoxins play a role in illness associated with damp and moldy buildings.

Other Health Effects: Other potential health effects following exposure to molds in contaminated buildings include pathogenicity and irritation. Except for chronic sinusitis (about 5 percent of the population) and dermatomycoses, most fungal infections are limited to immunocompromised individuals, including patients with AIDS, organ transplants, cancer, autoimmune diseases such as diabetes, or neonates. Irritation occurs in response to β -D-glucans and mVOCs produced by the fungi and bacteria growing indoors. Fungal volatile compounds may impact the “common chemical sense” which senses pungency and responds to it. This sense is primarily associated with the trigeminal nerve (and to a lesser extent the vagus nerve). This mixed (sensory and motor) nerve responds to pungency, not odor, by initiating avoidance reactions, including breath holding, discomfort, or paresthesias, or odd sensations, such as itching, burning, and skin crawling (Cometto-Muniz, Cain, 1993; Otto *et al.* 1990). Also, certain fungi can release highly toxic gases from the substrate on which they grow. For instance, one fungus growing on wallpaper released the highly toxic gas arsine from arsenic containing pigments (Gravesen *et al.* 1994).

Preliminary Risk-Assessment Models: Burge (1996) proposed a preliminary risk assessment model in 1996 that was based on LD50s. Since the primary exposure route in contaminated indoor environments is via inhalation, we recently proposed a risk assessment model, referred to as the “Inhalation Contact-Point Model” that is based on localized responses on the lung surface (Miller *et al.* 2000). Risk is then estimated based on the number of spores that are deposited per square inch of lung tissue area and/or the average amount of toxin per spore, which must be determined empirically. This latter model was proposed to help account for the elevated effects observed during chronic exposures than would be predicted from acute studies. The rat studies of Rao *et al.* (2000) demonstrated that spore depositions of as little as 20 to 40 spores/cm² induced acute pulmonary hemorrhage within 24 hours. It therefore seems feasible that chronic exposures could indeed lead to the pulmonary symptoms expressed in infants. Although the amount of estimated toxin in our example is extremely small, the localized toxic effect in the lung tissue surrounding the spores (ca. one spore per 10 square centimeters) could have dramatic effects on the health of the individual. This would not be evident without using the inhalation contact-point model. Hemolysin effects (Van Emon *et al.* 2003; Vesper, Vesper, 2002) would also be compatible with the contact point model.

CONCLUSIONS

Health effects following exposure to mold-contaminated environments include: irritation, allergic reactions, infection, and possibly intoxications. Of these, the effects of mycotoxins on human health in indoor environments remain controversial, but the preponderance of reports suggests that mycotoxins may indeed affect occupant health. Unfortunately, due to the lack of sensitive analytical methods combined with diffuse, often self-reported symptoms, risk assessments are reduced to qualitative rather than quantitative models. This results in a continued hotly-contested debate that is based, on both sides, on anecdotal data and conjecture, that can only be resolved with well-designed and objective scientific research.

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SUSTAINED FUNGAL CONTROL THROUGH INTERIOR FINISH PERFORMANCE REQUIREMENTS

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ABSTRACT

The interior finish plays a pivotal role in the quality of the indoor environment as related to effects of fungal growth. This impact originates from material constituents, but moreover from the ability of moisture storage and consequent prolongation of humidity conditions at the material surface that are favorable to fungal growth. The recognition of such crucial role calls for an approved newly developed method to assess materials fungal resistance, addressing the divergent behavior as a function of the moisture regime. Analysis of growth is based on the entire growth pattern as a function of time, including all stages of growth. Results underline that considering indoor climate dynamics is of major concern. In past years, pilot application of the new test on a wide range of finishing products led to an approved product qualification system with respect to fungal resistance, as a step towards performance requirements in building regulation.

INDEX TERMS: Interior finish, fungal resistance, test method, growth analysis, performance requirements.

INTRODUCTION

The interior finish plays a pivotal role in the quality of the indoor environment as related to effects of fungal growth. This impact obviously originates from material constituents, but moreover from the ability of moisture storage and consequent prolongation of humidity conditions at the material surface that are favorable to fungal growth (Adan, 1994). Particularly in modern highly insulated indoor environments, control of fungal growth is primarily a matter of material performance and ventilation is clearly a second-order effect. In this respect, growing ecological demands, environmental legislation, demographic trends and increasing energy conservation even enlarge pressure on performance requirements of finishing

materials. The industrial trend towards eco-friendlier building products is generally accompanied by an increase in constituent biodegradability.

The recognition of such crucial role calls for an approved method for assessing the interior finish performance in this context. Such method is a basic instrument for materials innovation and underlies the definition of material requirements and subsequent products labeling for end-user implementation.

METHODS

Present standards and methods to assess fungal growth: Basically, each test is designed to compare materials under the same conditions. Most standards cover a specific range of material only (sheet materials and plaster boards (Anon., 1968), paints and lacquers (Anon., 1989, 1986), film materials (Anon., 1975), textiles, printing wire materials (Anon., 1988 a, b) and apply a mixture of fungal species, intending to reflect the fungal flora in the considered field of application. A major drawback of using such mixture is that it obscures interpretation and analysis of growth in terms of discrimination and resolution (Adan, 1994).

Present methods use a single moisture regime and do not explicitly take account of effects of transient moisture loads and distinct material performance in relation to that. Most tests are based either on a more or less steady state level of the relative humidity below saturation (Anon., 1968, 1975, 1986, 1988 a) or non-unambiguous surface moistening (Anon., 1988b, 1989). Some tests prescribe natural or artificial soiling, for support of fungal spores to prevent removal by dripping water in the case of surface condensation (Anon., 1988 a, 1989), or to obtain indications of the efficacy of biocides in practice (Adan, 1994). It is obscure to what extent it reflects the actual domestic situation.

Reproducibility of such tests is highly affected by initial conditions. Inoculation is usually achieved by spraying an aqueous suspension of spores, however, without quantitative specification in terms of concentration and volume required. Variation in initial spores distribution and concentration may cause deviation in coverage area evolution. Undefined initial moisture conditions will cause uncertainties in growth rates in the initial stages (Adan, 1994).

And finally, in the majority of tests assessment takes place at the end of the test period only (Anon., 1968, 1975, 1988 a, b). Since it does not take account of growth pattern differences during the limited period of test, such approach may clearly lead to misinterpretation of material performance. Apparent differences may disappear or equal trends in growth may show divergent behavior if progress beyond the actual period of test is considered.

A new concept to assess fungal growth: A new method for assessment of materials resistance against fungal growth has been developed, giving follow-up to the recommendations of an International Workshop on fungi in indoor environments (Samson *et al.* 1994). The new test addresses the divergent behavior as a function of the moisture regime.

Exposure encloses both steady-state and transient conditions, i.e. an unsaturated (97%) relative humidity at 22 °C and an intermittent pattern of condensation at 35 °C and drying (RH < 60%) at 22 °C ambient temperature, respectively. Furthermore, *single* fungal species are used in both respective conditions, being predominant indicator organisms in W-European domestic environments: *Penicillium chrysogenum* Thom (CBS 401.92) in the former and *Cladosporium sphaerospermum* Penzig (CBS 797.97) in the latter.

The experimental arrangement consists of a closed re-circulating system, using counter flow humidification of the air, resulting in a defined relative humidity level with 1% RH inaccuracy above the material sample tested. Furthermore, a steady average air velocity below 5 cm.s⁻¹ is set, being typical for boundary flow along surfaces in the indoor environment.

Experimental design: Experimental design is based on a balanced set-up, i.e. replicate samples are included and positioned such that variance caused by position (local climatic distortions) can be determined. Considering statistics, a minimum of 6 replicates is recommended; from a practical point of view, aseptic conditions form a logical starting point. Despite this restriction, it has been shown that contamination is of no or minor effect (Adan, 1994; Adan *et al.* 1999). In the present experiments, 10 replicates have been used as standard.

Analysis of growth: Contrary to present day tests, analysis of growth is based on the *entire* growth pattern as a function of time, including all stages of growth. The analysis of growth includes the following successive steps.

First of all, assessment of fungal growth with the naked eye at repeated intervals during the period of test using the BS3900 (Anon., 1989) numerical scale (Table 1). During the assessment specimens remain in the incubator.

Table 1. Numerical scale to assess coverage area

Rating	Appearance
0	no growth
1	coverage $\leq 1\%$
2	$1\% < \text{coverage} \leq 10\%$
3	$10\% < \text{coverage} \leq 30\%$
4	$30\% < \text{coverage} \leq 70\%$
5	$70\% < \text{coverage}$

Secondly, mathematical modeling of the growth pattern is applied. The vegetative growth of both fungi considered on batch cultures produces sigmoidal curves. Adan (1994) introduced a non-linear regression technique to analyse the sigmoidal curve starting from the mathematical model $y_i = f(t_i, \mathbf{v}) + \xi_i$ where y_i is the rating for the coverage area at day i , f is a deterministic function depending on a vector \mathbf{v} of parameters to be estimated and ξ is a stochastic term that is assumed to be identically and independently normally distributed.

Numerous mathematical functions have been proposed for modeling sigmoidal curves, many of which are claimed to have some theoretical basis (Patten, 1971; Ratkowsky, 1983). Adan (1994) recommended a reparameterized logistic model reading:

$$(1) \quad y = \frac{\alpha}{1 + e^{4\varepsilon \cdot (\delta - t)/\alpha}}$$

where α is the upper asymptotic value of y , δ the time coordinate of the inflection point and ε the first derivative with respect to time in the inflection point. α actually represents the estimated final coverage area; δ can be considered a measure for the growth rate in the exponential stage, i.e. the highest growth rate occurring at the moment of time ε . The logistic model is a nonlinear model, because the coefficients δ and ε to be estimated appear non-linearly.

Given the assumption of independent and identically distributed normal variables, the criterion of least squares in linear models provides the best available estimates in practice. Nonlinear models tend to do so only as the sample size becomes very large. In addition, the distributional properties of the stochastic term are often unknown. In simulations of Ratkowski (1983) the coefficient estimates in the logistic model, however, remained stable to various assumptions about the error term. Furthermore, the nonlinear behavior of the coefficients is not serious in practical terms, as simulations showed that the distribution of the coefficient estimators approached that expected for the normal distribution.

Such growth pattern modeling allows a distinct evaluation of growth in terms of response variables. The disadvantage of assessment with the naked eye is that it introduces non-linearity. Although analysis of the observer variability revealed high inter-observer agreement (Adan, 1994), data acquisition is highly dependent on the human factor in terms of continuity. Recent application of image analysis techniques showed prospects to tackle these drawbacks and to improve resolution even more (to be published).

Pilot application on a wide range of materials: During the past decade, the fungal resistance of a wide variety of materials has been assessed using the proposed method of test. Main objectives of this pilot application were to:

- determine whether the test allows sufficient discrimination between materials,
- check the reproducibility in various application areas,
- optimize the test with respect to these issues,
- make the step towards an approved product qualification system

The following materials and products were subjected to this pilot research: silicon caulking, typically applied in sanitary rooms (Adan, Lurkin, 1997 a); a wide range of coating types, including waterborne interior paints (Adan *et al.* 1999); specialties, such as high-absorbing claddings (Adan, Lurkin, 1997 b) and ceramic coatings (Sanders, 2002 a); fiber products; gypsum-based plasters, wall papers, including glues (Adan *et al.* 1999); cement-based panels (Sanders, 2002 b). For some products, affected spots in practice obviously show known selectivity in terms of fungal species. Therefore, in case of silicon caulking, the test included *Aureobasidium pullulans* var. *melanogenum* (CBS 621.80), *Phoma herbarum* (CBS 366.61) and the yeast *Rhodotorula glutinis* (CBS 2890), mirroring the common microflora predominantly occurring on this type of material in bathrooms (Reenen-Hoekstra *et al.* 1991; Hoekstra, personal communication).

Generally, the logistic model provided a satisfactory fit to the data, as the coefficient of determination $R^2 > 0.96$. The estimator proved to be the most appropriate tool to compare fungal resistance, with a satisfactory reproducibility (Adan, Lurkin 1997 a; Adan *et al.* 1999). No adventitious contaminants have been observed covering the sample surfaces.

Tests revealed a significant range of (varying between 0 and 5) within a test period of 3 months, evidently discriminating between different products. Furthermore, the experiments showed that growth might be clearly dependent on moisture loads, i.e. behavior under steady state and transient conditions may highly differ. Assessment of growth at a distinct moment in time only, as with most of the pres-

ent standards, would have obscured the observed differences originating from the present growth pattern analysis.

RESULTS

Towards performance requirements of interior finishing products: A sustained control strategy of indoor fungal growth should consider the pivotal role of finishing products. Generally, the pilot application during the past decade yielded a highly reproducible and discriminating picture of material performance in terms of fungal resistance, and, moreover, showed performance that might highly differ as related to the moisture load. In other words: fungal resistance is a *product*-based feature, and *application-oriented*, underlining that consideration of indoor climate dynamics is of major concern. These statements laid a foundation for an approved product qualification system in the Netherlands with respect to fungal resistance. Such system is a step towards performance requirements in building regulation, making it compulsory, but moreover, product labeling provides simple decision-support to end-users, i.e. tenants or building owners, the actual occupants.

Labeling is defined according to a 3 level classification system, introducing the classes 'resistant' and 'sensitive', and an intermediate level 'fairly resistant' (see Table 2). These definitions are based on threshold values of the estimator α , since this variable proved to be the most appropriate and discriminating tool to compare product performance. The estimator of δ appeared less suitable, as it usually shows higher variance originating from the logistic fit. δ showed to be valuable to refine assessment in the frame of product innovation. The threshold value of α is founded on the overall picture provided by the data set of the pilot study. These data, covering a wide range of materials, showed α ranging between 0-1 or 3-5.

The basic principle underlying the classification is the potentiality of most products to exhibit a widely divergent behavior as a function of the moisture load. In the past decade, in about 50 % of the tested products, the estimators α_{steady} and α_{con} for the steady-state and transient (i.e. condensation) conditions, respectively, were on different sides of the threshold values, underlining the relevance of both tests to assess product performance. Consequently, the labeling system should be directly connected to the overall characteristics of the indoor humidity load, i.e. to a recommended application. The best quality (I) in terms of resistance reflects the fact that the majority of mold problems occurs in indoor areas with a distinct vapor production (e.g. bathrooms and kitchens in 60 and 40% of cases in the Netherlands, respectively (Anon., 1993). Logically, it is principally based on α_{con} . Under these circumstances, the finishing product is truly dominating the risks for fungal growth, as has been shown fundamentally (Adan, 1994). In all other indoor areas, with a more or less steady state indoor humidity, risks of surface growth are

a consequence of interaction of finishing product, building construction -thermal bridging in particular-, and average humidity or ventilation. In that case, product labeling is based on the α_{steady} and discriminates between fairly resistant products that can be applied on thermal bridges, and sensitive products that should be applied on inner constructions in dry environments only.

Table 2. The Dutch classification system for fungal resistance of interior finishes (α_{con} and α_{steady} represent the estimators for the transient and steady-state conditions, respectively)

Class	Quality	Recommended application	Definition
I	Resistant	Indoor environments with transient moisture loads such as bathrooms, kitchens, production processes, swimming pools	$\alpha_{\text{con}} \leq 1.25$
II	Fairly resistant	All other indoor areas, with a more or less steady state indoor humidity, such as living rooms, attics, storage rooms or depots	$\alpha_{\text{con}} > 1.25$ $\alpha_{\text{steady}} \leq 1.25$
III	Sensitive	Only on inner constructions not being part of the building envelope in environments other than class I.	$\alpha_{\text{con}} > 1.25$ $\alpha_{\text{steady}} > 1.25$

CONCLUSION

In terms of fungal resistance, the pilot application clearly revealed that fungal resistance can be considered a product feature and usually is not a generic characteristic of a type of material. Nonetheless, until now some types appear to show uniformity, e.g. waterborne paints tested were all labeled III.

ACKNOWLEDGEMENTS

The authors greatly acknowledge the Stichting Bouwresearch Rotterdam that partly funded the development of a product labeling system for end user implementation. Furthermore, they are indebted to Mrs. J. H. M. Lurkin, who contributed to the experimental work during the period of 1995-2000.

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Appendix

Fotos & Index