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Timo Murtoniemi

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Department of Environmental Health
National Public Health Institute, Kuopio, Finland
and
Department of Biochemistry, University of Kuopio, Finland

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MICROBIAL GROWTH ON PLASTERBOARD AND SPORE-INDUCED CYTOTOXICITY AND INFLAMMATORY RESPONSES *IN VITRO*

Timo Murtoniemi

National Public Health Institute
Department of Environmental Health
P.O.Box 95
FIN-70701 Kuopio, Finland

and

University of Kuopio
Department of Biochemistry
P.O.Box 1627
FIN-70211 Kuopio, Finland

ACADEMIC DISSERTATION

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Publisher: National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki, Finland
Phone +358 9 47441
Telefax +358 9 4744408

Author's address: National Public Health Institute
Department of Environmental Health
P.O.Box 95, FIN-70701 Kuopio, Finland
Phone +358 17 201369
Telefax +358 17 201155
E-mail timo.murtoniemi@ktl.fi

Supervisors: Docent Maija-Riitta Hirvonen, Ph.D.
National Public Health Institute
Kuopio, Finland

Docent Aino Nevalainen, Ph.D.
National Public Health Institute
Kuopio, Finland

Reviewers: Professor Atte von Wright
University of Kuopio
Institute of Applied Biotechnology
Kuopio, Finland

Professor Tari Haahtela
Hospital District of Helsinki and Uusimaa
Skin and Allergy Hospital
Helsinki, Finland

Opponent: Professor Tiina Reponen
University of Cincinnati
Cincinnati, Ohio, USA

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ABSTRACT

Moisture damage and microbial growth in buildings have been associated with health effects among the occupants including various respiratory symptoms and asthma. However, the causative agents and the underlying mechanisms that are responsible for the adverse health effects associated with exposure in buildings are not clear. The basic phenomenon of microbial growth on building materials must be better understood. One possibility is that the growth conditions of microbes on wetted building materials are different from the natural environment, and this may encourage the microbes to produce biologically active compounds. In this thesis the growth of four microbes *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium spinulosum*, and *Streptomyces californicus* isolated from moisture damaged buildings was studied on six commercially available plasterboards and separately on the liners and cores of those boards. The biological activity of the spores was assessed as the ability of the spores to induce cytotoxicity and the production of pro-inflammatory cytokines and nitric oxide in mouse macrophages. Moreover, the effects of plasterboard composition and the use of a biocide on microbial growth and subsequent biological activity of spores were tested.

There were considerable differences between commercially available plasterboards in their ability to support microbial growth with subsequent production of biologically active compounds capable of inducing inflammatory and toxic reactions in mammalian cells. These microbiological and immunological reactions depended also on the microbial species. *Stachybotrys chartarum* grew consistently faster than the other studied microbes whereas the spores of *Streptomyces californicus* were the most potent inducers of inflammatory responses. In some cases, microbial growth and biological activity of spores were more abundant on cores than on liners.

The studies of plasterboard compositions revealed that growth of both *S. chartarum* and *Str. californicus* decreased compared to reference board in those cases where (a) the liner was treated with a biocide, (b) starch was removed from the plasterboard, or (c) desulforisation gypsum (DSG) was used in the core. Addition of the biocide into the core inhibited the growth of *Str. californicus* almost completely but did not reduce the growth of *S. chartarum*. In fact the spores of *S. chartarum* collected from that board evoked the highest detected cytotoxicity. Removal of starch reduced the bioactivity of *Str. californicus* spores but it did not affect that of *S. chartarum* spores.

Altogether, these results suggest that microbial growth on plasterboard and subsequent biological activity of spores is not only due to the paper liner of plasterboard, but the core material also has a crucial role. The microbial growth and biological activity of spores can be affected by minor changes to the composition of core or liners but it cannot be totally prevented without resorting to the use of biocides. However, the use of biocides has to be carefully evaluated since incomplete prevention of microbial growth by biocides may even increase the harmfulness of the microbial spores.

To Anja and Pentti

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Timo Murtoniemi

ABBREVIATIONS

A. versicolor	*Aspergillus versicolor*
AOC	assimilable organic carbon
AODC	acridine orange direct counting
a_w	water activity
$CaSO_4 \cdot 2H_2O$	calcium sulfate dehydrate (gypsum)
DNA	deoxyribonucleic acid
DSG	desulforisation gypsum
ELISA	enzyme-linked immunosorbent assay
FAME	fatty acid methyl ester
GC-MS	gas chromatography equipped with a mass selective detector
HBSS	Hank's balanced salt solution
Ig	immunoglobulin
IL	interleukin
INF	interferon
IPH	idiopathic pulmonary hemosiderosis
LPS	lipopolysaccharide
MEA	malt extract agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,3-dimethyltetrazolium bromide
NF	nuclear factor
NG	natural gypsum
NO	nitric oxide
NOS	nitric oxide synthase
P. spinulosum	*Penicillium spinulosum*
rDNA	ribosomal DNA
RH	relative humidity
ROS	reactive oxygen species
S. chartarum	*Stachybotrys chartarum*
SDS	sodium dodecyl sulfate
Str. californicus	*Streptomyces californicus*
Th	helper T lymphocyte
TMS	trimethylsilyl
TNF	tumor necrosis factor
TOC	total organic carbon
TYG	tryptone - yeast extract - glucose
VOC	volatile organic compound

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by the Roman numerals I-V.

I Murtoniemi, T., Nevalainen, A., Suutari, M., Toivola, M., Komulainen, H. and Hirvonen, M.-R. (2001). Induction of cytotoxicity and production of inflammatory mediators in RAW264.7 macrophages by spores grown on six different plasterboards. *Inhal Toxicol*, 13, 233-247.

II Murtoniemi, T., Hirvonen, M.-R., Nevalainen, A. and Suutari, M. (2003). The relation between growth of four microbes on six different plasterboards and biological activity of spores. *Indoor Air*, 13, 65-73.

III Murtoniemi, T., Nevalainen, A., Suutari, M. and Hirvonen, M.-R. (2002). Effect of liner and core materials of plasterboard on microbial growth, spore-induced inflammatory responses and cytotoxicity in macrophages. *Inhal Toxicol*, 14, 1087-1101.

IV Murtoniemi, T., Nevalainen, A., and Hirvonen, M.-R. (2003). Effect of plasterboard composition on *Stachybotrys chartarum* growth and biological activity of spores. *Appl Environ Microbiol*, in press

V Murtoniemi, T., Keinänen, M. M., Nevalainen, A., and Hirvonen, M.-R. (2003). Starch in plasterboard sustains *Streptomyces californicus* growth and bioactivity of spores. *J Appl Microbiol*, 94, 1059-1065.

TABLE OF CONTENTS

1 INTRODUCTION

Various health complaints have often been attributed to pollution and the poor quality of the indoor air during the past few decades. There are several different factors that contribute to the indoor environment. Inappropriate temperature and dry air as well as insufficient ventilation of the building leading to increased concentrations of CO_2 are typical causes for discomfort among occupants. Indoor air may also contain pollutants such as inhalable particles, radon, inorganic dusts, volatile compounds emitted from building materials, and allergens derived from mites, pets and vermin. Microbial growth on building materials as a result of excess moisture may also affect the indoor air quality.

If there is good practice in building design, construction, and maintenance, the structures should remain dry. However, when there is moisture damage, the materials may be in contact with water for prolonged times, which inevitably leads to microbial growth or even total deterioration of the building materials (Oliver, 1997). Moisture and mold problems are relatively common in modern buildings. Studies from different countries have reported prevalences of dampness and microbial growth in buildings ranging from 20 % to 55 % (Platt *et al.*, 1989; Dales *et al.*, 1991; Brunekreef, 1992, Nevalainen *et al.*, 1998). Moisture damages typically occur due to construction defects or errors as well as aging or corrosion of building materials and lack of maintenance (Chelelgo *et al.*, 2001). The most common sources for moisture in buildings can be divided into four categories: 1) Leakage of water originating from rain and snow or rising moisture from the ground. 2) Moisture within building materials and constructions, *e.g*. humidity in concrete floors or condensation on cold surfaces due to poor ventilation or insufficient insulation. 3) Leakage of water by accident, *e.g*. from plumbing. 4) Moisture from human and indoor activities, *e.g*. human expiration, laundry, cooking, and bathing (Bornehag *et al.*, 2001).

Moisture damages and microbial growth in buildings have been associated with adverse health effects experienced by the occupants in several epidemiological studies (Verhoeff and Burge, 1997; Peat *et al.*, 1998; Bornehag *et al.*, 2001). Currently, the exposing agents and underlying mechanisms leading to these health outcomes are still poorly understood. It is evident, however, that the microbial growth phenomena and subsequent exposure to microbes

and their products are responsible for the reported symptoms. Therefore, the characteristics of the building materials to act as substrates for microbial growth have to be better understood.

Plasterboard is a commonly used building material. It is annually used over 2 billion square meters worldwide. In the manufacture of plasterboard, a core of gypsum plaster is enclosed between two heavy paper liners in a continuous process. Over 90 % of plasterboard weight is composed of gypsum which is chemically defined as calcium sulfate dehydrate ($CaSO_4 \cdot 2H_2O$). The gypsum used in plasterboards may be of natural origin or it may have been obtained from a process, which removes sulfur dioxide from power station flue gases (desulforisation gypsum). In addition to gypsum, plasterboard core also contains water and some additives, such as starch. The use of gypsum by the construction industry is attributable to its many favorable technical characteristics such as processability, lightweight, resistance to fire, and reduction of sound. On the other hand, excess water could be absorbed by capillary movement into gypsum and that may serve as a reservoir of water and nutrients, favoring conditions for microbial growth (Pasanen *et al.*, 2000b).

In cases of moisture damage, wetted plasterboards, like almost any building material, have been found to be contaminated with microbes (Nielsen *et al*., 1998a; Hyvärinen *et al*., 2002). It has been previously shown that the building material on which the microbe is growing markedly affects the ability of the microbe to induce inflammatory responses *in vitro* (Roponen *et al.*, 2001). In that study, plasterboard was one of the building materials that induced the highest responses in mammalian cells. This is in line with several other studies which have suggested that inflammation is a possible pathophysiological mechanism behind many of the health effects related to exposure to indoor air microorganisms (Nielsen *et al*., 1995).

In this thesis, the microbial growth on plasterboards was studied with four microbial strains representing the species *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium spinulosum*, and *Streptomyces californicus*. These are all microbes that are commonly detected in moisture damaged environments (Hyvärinen *et al*., 2002). The cytotoxic and inflammatory potential of the spores collected from the growth on the plasterboards was evaluated by analyzing their ability to induce cytotoxicity and the production of important inflammatory mediators in mouse macrophages.

2 REVIEW OF THE LITERATURE

2.1 Health effects related to moisture damage and microbial growth in buildings

Several epidemiological studies in different countries have reported an association between dampness and microbial growth in buildings and adverse health effects among the occupants. The symptoms are typically related to inflammatory reactions, such as lower respiratory symptoms (cough, phlegm, wheeze) (Dales *et al.*, 1991; Brunekreef, 1992; Spengler *et al.*, 1994), respiratory infections (Waegemaekers *et al.*, 1989; Koskinen *et al.*, 1999), and allergic diseases, including allergic asthma (Garrett *et al.*, 1998; Kilpeläinen *et al.*, 2001; Lander *et al.*, 2001). Non-inflammatory, unspecific symptoms, such as eye and skin irritation, fatigue, headache, nausea, and vomiting have also been reported (Platt *et al.*, 1989; Koskinen *et al*, 1999). The symptoms usually decrease after cessation of the exposure (Koskinen, 1995, Johanning *et al.*, 1999; Meklin *et al.*, 2003). However, the exposure levels to microbes in indoor environments have not been especially high (Nevalainen *et al*., 1991; Hyvärinen *et al*., 1993 & 2001). Thus, the agents and mechanisms whereby the exposure in moisture damaged indoor environments can lead to these symptoms are not clear.

Severe pulmonary diseases have also been reported among the occupants in moisture damaged buildings (Husman, 1996) although they are usually related to high occupational exposure levels *e.g.* in agriculture or in sawmills (Mandryk *et al*., 2000; Eduard *et al*., 2001). Certain fungal genera such as *Aspergillus* or *Penicillium* that are common colonizers in water damaged buildings have also been shown to cause invasive opportunistic infections especially among immunocompromised patients (Denning, 1998; Lyratzopoulos *et al.*, 2002).

2.2 Possible causative agents of the health effects

Airborne microbial particles consisting of spores, fragments, or cells have been suggested to be possible causative agents of the health effects experienced by occupants of moisture damaged buildings (Cooley *et al.*, 1998; Garrett *et al.*, 1998; Jacob *et al.*, 2002; Gorny *et al.*, 2002). In those buildings, the concentrations of viable microbes in the indoor air are usually

relatively low, of the order of magnitude 10-10^3 cfu/m^3 (Nevalainen *et al*., 1991; Hyvärinen *et al*., 1993 & 2001) compared to high exposure levels in occupational environments, often 10^4-10^6 cfu/m^3 (Mandryk *et al*., 2000; Eduard *et al*., 2001). However, the composition of microbial flora in moisture damaged buildings typically differs from that of non-damaged buildings (Hyvärinen et al., 2001) suggesting that also the distinct characteristics of the exposure can affect the health of the occupants of moisture damaged buildings.

Spores are mycelial-derived structures of filamentous fungi and some bacteria that are produced to aid in the dispersal of their genetic material. Spores are more resistant to environmental stress than mycelia and thus they can also be viewed as survival forms of microorganisms. The release of spores into the air is dependent on the microbial genus as well as on different environmental factors including air velocity and relative humidity (Pasanen *et al.*, 1991; Reponen *et al.*, 1998), texture of the surface, and vibration of the contaminated material (Gorny *et al.*, 2001). Recent studies have shown that fungal or bacterial fragments that are considerably smaller in size than the actual spores aerosolized simultaneously from contaminated surfaces (Gorny *et al.*, 2002 & 2003). It has been suggested that these fragments are derived from pieces of mycelium, spores, or fruiting bodies. They may also be formed through nucleation from secondary metabolites of fungi (Gorny *et al.*, 2002).

Exposure to microbial particles may occur via those tissues that are in direct contact with the air including airways, eyes, and skin (Mølhave, 1998). With respect to the respiratory symptoms, inhalation is the most relevant absorption route. The aerodynamic particle size is a critical factor, determining the extent of respiratory exposure as well as site of deposition in the airways and hence the type of possible respiratory reactions provoked by the microbial particles. The aerodynamic sizes of fungal spores typically range from 2 μ to 10 μm whereas bacterial spores and vegetative cells are smaller with a size of approximately 1 μm (Reponen et al., 2001). The larger particles (> 5 μm) settle faster whereas the smaller particles (< 5 μm) remain airborne for longer periods when they can be inhaled (Reponen, 1995). Consequently, larger particles react in the upper airways whereas smaller particles are small enough to reach the alveoli.

Sensitization to fungal allergens has been shown to increase atopic symptoms and the severity of asthma (Jacob *et al.*, 2002; Zock *et al.*, 2002; Zureik *et al.*, 2002). This suggests that an IgE mediated allergic reaction to fungi may play a role in indoor microbial exposure and respiratory symptoms. However, fungal allergy among children exposed in moisture damaged schools has been determined to be rather rare (Taskinen *et al.*, 1997; Immonen *et al.*, 2000), suggesting that mechanisms other than allergy are also involved.

Several structural components of microbes such as endotoxin (LPS) of gram-negative bacteria or fungal cell wall components, (1→3)-β-D-glucans, have been shown to possess inflammatory potential *in vitro* (Fogelmark *et al*., 1994; Thorn and Rylander, 1998; Douwes *et al.*, 2000). This suggests that exposure to these kinds of components in moisture damaged buildings may also impact detrimentally on health.

A number of fungi, such as the genera *Stachybotrys*, *Aspergillus*, *Fusarium*, and *Trichoderma* may produce toxic secondary metabolites, which are generally called as mycotoxins (Nielsen *et al*., 1998a,b; Jarvis, 2002; Nieminen *et al.*, 2002). Similarly, toxic substances produced by bacteria such as the genera *Streptomyces* and *Bacillus* are called bacterial exotoxins (Andersson *et al.*, 1998, Peltola *et al*., 2001a,b). Microbial toxins in moisture-damaged buildings have been associated with non-allergic general symptoms (Croft *et al.*, 1986), with respiratory disorders, such as idiopathic pulmonary hemosiderosis (IPH) in infants (Dearborn *et al.*, 1999) and impaired lung function in adults (Hodgson *et al.*, 1998). The effects of mycotoxins have mainly been studied in veterinary or in animal models. These studies have revealed that mycotoxins can evoke a multitude of toxic effects including acute toxicity and immunosuppression (Jarvis, 2002). When mycotoxins have been found in moisture damaged buildings, they have usually been detected from material samples rather than from the indoor air (Hodgson *et al.*, 1998; Nielsen *et al*., 1998a; Tuomi *et al.*, 2000). Therefore, the route of exposure to mycotoxins and thus the causal relationship between the toxins and health effects in moisture damaged buildings remains obscure.

Microbes produce a wide variety of volatile organic compounds (VOC) depending on the growth medium and the microbial species (Sunesson *et al.*, 1996; Korpi *et al.*, 1998 & 1999). Microbial VOCs are responsible for musty odors and may cause non-specific, irritative symptoms (Wolkoff and Nielsen, 2001).

Altogether, the exposure in moisture-damaged buildings consists of both particles and volatile compounds and it is likely that the adverse health effects are due to multiple factors.

2.3 Factors affecting microbial growth in buildings

The most important factor regulating microbial growth in indoor environments is the amount of available water in the building material, defined as water activity (a_w). Microbial growth is likely if the a_w of a material exceeds 0.76-0.96, depending on the microbial species, temperature and availability of nutrients (Grant *et al.*, 1989; Pasanen *et al.*, 1992). Grant *et al.* (1989) found that fungi appear on gradually moistening building materials in a succession based on the moisture requirements of the fungal species, *i.e.* the primary colonizers come first (a_w < 0.80, *e.g. Penicillium*), followed by secondary colonizers (a_w 0.80-0.90, *e.g. Aspergillus versicolor*) and tertiary colonizers (a_w > 0.90, *e.g. Stachybotrys chartarum*). At low temperatures or when the materials have a restricted nutritive capacity, the required moisture is higher (Grant et al., 1989). This may usually be the case in building materials consisting of non-organic constituents. Under fluctuating humidity and temperature conditions, microbial growth is slower compared to stable moist conditions (Pasanen *et al.*, 2000a). In general, low a_w usually favors sporulation, whereas mycelial growth is more likely at high a_w (Cahagnier *et al.*, 1993). It has been shown that microbes survive better on building materials under slow-drying conditions (Korpi *et al.*, 1998) than if the material dries quickly (Chang *et al.*, 1995).

The relative humidity (RH) of air has only an indirect influence on microbial growth. High RH increases condensation of water on cold surfaces but fungi can also grow at very low RH if the moisture content of the material is high enough (Pasanen *et al.*, 2000b). At a low RH, spore liberation into the air is increased (Gravesen et al., 1994) which may also affect the concentration, and thus, the exposure levels to the fungi.

The temperature in buildings usually favors mesophilic microbes that grow in the temperature range 5-35^{o}C, with an optimum temperature between 20-25^{o}C. Most fungi will tolerate a wide

range of pH but prefer neutral conditions between pH 5-6 (Dix and Webster, 1995), which also is the pH existing in most building materials.

In addition to water and suitable temperature and pH conditions, microbes require sources of carbon and nitrogen, a supply of energy and certain essential nutrients such as potassium and phosphorus. Several trace metals such as Cu, Zn, Fe, Ni, and Mn at low concentrations are also critical for microbial growth. However, the supply of these metals is likely to be achievable on most building materials. On the other hand, heavy metals such as Pb, As, Cd, and Al may be strongly inhibitory for microorganisms even at relatively low concentrations (Gadd, 1990). Various building materials clearly have different substrate characteristics with which to support microbial growth (Hyvärinen, *et al*., 2002). In order to obtain these substances from their growth environment, microbes secrete a wide variety of enzymes into the extracellular fluid (Griffin, 1994).

When the growth limiting nutrients are exhausted or the microbes are having to compete with other microorganisms, microbes undergo a cascade of metabolic changes permitting the adaptation to stress conditions and giving advantage against their competitors. Under these conditions, organisms enter a period of slower growth and undergo morphological alterations and changes in metabolism known as secondary metabolism (von Döhren and Gräfe, 1997). The majority of bioactive products of microorganisms, including antibiotics, mycotoxins, pigments, and enzyme-inhibitors are generated during secondary metabolism (Griffin, 1994). It has been shown that there is a complex interaction between a_w, temperature, duration of growth, and substrate availability on the production of secondary metabolites (Cochet and Demain, 1996; Gqaleni *et al.*, 1997)

Microbial growth on building materials may be reduced by using various biocides (Shirakawa et al., 2002). Biocides can be produced synthetically or they may be of natural origin produced by bacteria, fungi, plants, and animals. Different microbial genera have been shown to have considerable variation in their sensitivity to biocides (Russel, 2002). The selective toxicity of biocides depends on the target sites and the resistance mechanisms. Target sites of biocides may be functional groups of enzymes, cell membranes and lipid metabolism, energy production, nucleic acid and protein metabolism, cell wall biosynthesis, or nuclear division. Correspondingly, resistance to biocides may be acquired by genetic mutation or physiological

adaptation (Griffin, 1994). The variation in effectiveness of biocides against different microorganisms suggests that it may not be possible to completely prevent the microbial growth on building materials and thus their incorporation should be carefully considered and tested.

2.4 Microbes found from wetted plasterboards

In general, if there is enough moisture present, microbial growth occurs on all building materials, even on inorganic ceramic products (Hyvärinen *et al.*, 2002), since the deposition of dust and dirt may serve as nutrients for many microbes. However, the building materials most susceptible for microbial growth are usually wetted and aged organic materials containing cellulose, such as wooden materials, jute, and paper (Gravesen *et al.*, 1999; Hyvärinen *et al.*, 2002). The diversity of fungal genera growing on wetted plasterboards have been shown to be low compared to *e.g*. wooden materials or mineral insulation (Hyvärinen *et al.*, 2002). The most common fungal genera detected from water-damaged plasterboards include *Penicillium*, *Stachybotrys*, *Aspergillus*, *Acremonium*, *Sphaeropsidales*, and *Aureobasidium* (Gravesen *et al.*, 1994; Pasanen *et al.*, 2000b; Hyvärinen *et al.*, 2002).

In the following section, the four microbial species that were used in this thesis are presented. They were selected for these studies to represent different types of microbes commonly isolated from moisture damaged buildings.

Penicillium spp. The genus *Penicillium* consists of a wide number of species. They typically grow rapidly and produce large numbers of dry and small spores (Miller, 1992). Some species can also produce antibiotics and mycotoxins (Griffin, 1994; Nielsen *et al*., 1999). *Penicillium* is among the most frequently found fungal genera in the indoor air (Hyvärinen *et al*., 1993 & 2001). It is also the most common colonizer on water-damaged plasterboards (Gravesen et al., 1999; Hyvärinen *et al.*, 2002).

Stachybotrys chartarum. *Stachybotrys chartarum* is a cellulose-decomposing fungus that grows well on cellulose rich substrates such as straw and hay which also support the production of several mycotoxins (Hintikka, 1977) and proteinases (Kordula *et al.*, 2002). When growing on water-damaged building materials including plasterboards, *Stachybotrys*

species have been shown to produce highly toxic macrocyclic trichothecenes, *e.g.* satratoxins G and H (Nikulin *et al.*, 1994; Andersson *et al.*, 1997; Nielsen, *et al.*, 1998 a, b; Flappan *et al*., 1999; Tuomi *et al.*, 2000). However, two-thirds of the *S. chartarum* isolates produce atranones and simple trichothecenes that are much less cytotoxic than the macrocyclic trichothecenes (Nielsen *et al.*, 2001; Jarvis, 2002). Nikulin *et al.* (1994) detected a strong growth of *S. chartarum* on wallpaper, plasterboard, hay, and straw under saturated conditions with subsequent production of satratoxins G and H. In contrast, on pine panels, no toxic effects were observed, even though the growth of *S. chartarum* was extensive. This suggests that the ability of *S. chartarum* to produce these toxic metabolites depends on both the strain and the growth substrate.

Aspergillus versicolor. *Aspergillus versicolor* is a frequently found colonizer in various water damaged building materials including plasterboard (Hyvärinen *et al.*, 2002). It is a potent producer of several secondary metabolites such as cyclopenin, cyclopenol and versicolorin, which have strong antibacterial and antifungal activities (Gravesen et al., 1994). When grown on wetted building materials or in the carpet dust of mold problem buildings, *A. versicolor* has also been shown to produce sterigmatocystin, which is a precursor of the carcinogenic aflatoxins, (Nielsen *et al.*, 1998b; Tuomi *et al.*, 2000; Engelhart *et al.*, 2002).

Streptomyces spp. are a group of gram-positive bacteria typically found in soil. They are well known for their potential to produce a variety of different biologically active secondary metabolites such as antibiotics (Kutzner, 1986). Their presence in buildings indicates increased moisture in the structures providing conditions for microbial growth (Nevalainen *et al.*, 1991; Samson *et al*., 1994). Species of *Streptomyces* have also been shown to grow on contaminated plasterboards, often simultaneously with *Acremonium* and *Aspergillus* spp. (Hyvärinen *et al.*, 2002). Toxic valinomycin producing *Streptomyces griseus* strains have also been isolated from moisture damaged buildings (Andersson *et al.*, 1998).

2.5 Airway inflammation

The symptoms in moisture damaged buildings are usually associated with inflammatory reactions (Dales *et al*., 1991; Nielsen, 1995). Inflammation is a process with a complex series

of reactions executed by the host to prevent ongoing tissue damage, *e.g.* attempts to isolate and destroy the infective organism and to activate the repair processes to restore the organism to normality (Baumann and Gauldie, 1994). The clinical signs of inflammation are classically described as rubor (redness), tumor (swelling), calor (heat), and dolor (pain). The cellular events causing inflammation result from increases in vascular flow, induction of major changes in the endothelium, and the migration of leukocytes from the circulation to the site of injury with subsequent activation of the inflammatory cells (Lyons, 1995).

Alveolar epithelial cells and macrophages are the first line of defense against inhaled allergens, infectious agents and other immunological stimuli in airway inflammation (Lukacs *et al.*, 1995; Thomassen and Kavuru 2001; Haddad, 2002). Epithelial cells function as a physical barrier against foreign particulate matter whereas macrophages can also phagocytose (ingest) and eliminate the foreign matter. In addition, both of these cell types may amplify an ongoing inflammation by producing a variety of inflammatory mediators including cytokines, leukotrienes, growth factors, and reactive oxygen species.

2.5.1 Inflammatory mediators

2.5.1.1 Cytokines

The inflammatory response against inflammatory stimuli is a complex, dynamic, and strictly regulated process involving a balance between pro- and anti-inflammatory mechanisms. One of the most important factors in the regulation and persistence of inflammation at a local level in the tissue is a group of low molecular weight, soluble proteins, called the cytokines. Typical cytokines participating in airway inflammation and their main functions are presented in Table 2.1 and in Figure 2.1.

Table 2.1. Typical cytokines participating in airway inflammation and their main functions.

Cytokine	Main source	Function	Reference
IL-1β	Macrophages	Neutrophil activation T cell activation ↑Cytokines (e.g. IL-6, TNFα) ↑NO ↑Acute phase proteins ↑Adhesion molecules ↑Fever	Baumann and Gauldie, 1994 Barnes *et al.*, 1998
IL-4	Th2 cells	B cell activation ↓IL-1β, TNFα, IL-12 ↑Basophils, Eosinophils	Barnes *et al.*, 1998 Cenci *et al.*, 1999 Alexis *et al.*, 2002
IL-6	Macrophages Epithelial cells T cells	Neutrophil activation T cell activation Microbicidal activity Differentiation of B cells ↑Fever ↓IL-1β and TNFα ↑Glucocorticoids	Baumann and Claudie, 1994 Barton, 1997 Barnes *et al.*, 1998 Cenci *et al.*, 2001
IL-8	Macrophages Epithelial cells T cells	Neutrophil chemoattractant	Barnes *et al.*, 1998
IL-10	Th2 cells	Macrophage inhibition ↓IL-1β, TNFα, IL-6, IL-12 ↓NO Th1 cell inhibition	Barnes *et al.*, 1998 Murphy *et al.*, 1998 Cenci *et al.*, 1999
IL-12	Macrophages B cells	Cytotoxic T cell activation ↑INFγ, TNFα Th2 cell inhibition ↓IgE	Barnes *et al.*, 1998 Murphy *et al.*, 1998
INFγ	Th1 cells	Macrophage activation ↑IL-1β, TNFα Th 2 cell inhibition	Barnes *et al.*, 1998 Mosmann and Sad 1996
TNFα	Macrophages	Neutrophil activation T cell activation Antifungal activity Cytotoxicity ↑Cytokines (e.g. IL-1β, IL-6) ↑NO ↑Fever ↑Acute phase proteins	Fiers, 1991 Baumann and Claudie, 1994 Barnes *et al.*, 1998 Engele *et al.*, 2002

IL, interleukin; INF, interferon; TNF, tumor necrosis factor; NO, nitric oxide

Tumor necrosis factor alpha (TNFα) is a cytokine that is well known because of its cytotoxic and antitumoral activity (Fiers, 1991). It is now clear that it also exerts biological effects on many different cell types and it has an important role in several physiological and pathological conditions *e.g.* as mediator of sepsis (Beyaert and Fiers, 1994, Ayala *et al.*, 2003). With respect to airway inflammation, TNFα along with interleukin (IL)-1β are pro-inflammatory cytokines that are mainly produced by airway macrophages, as part of the innate, non-specific immune response against inflammatory stimulus such as microbial infections. These cytokines activate vascular endothelium and increase vascular permeability, enhancing access of the effector cells to the site of the inflammation (Sedgwick *et al.*, 2002). They stimulate neutrophil proliferation, cytotoxicity, production of proteolytic enzymes, and activation of lymphocytes. They also activate airway epithelial cells to initiate a cascade of the so called "second wave" cytokines including IL-8 and IL-10 and other inflammatory mediators that modify the inflammatory responses to either amplify or attenuate the inflammation (Kelley, 1990; Cluezel and Lee, 1992; Barnes *et al.*, 1998).

IL-6 is also regarded as a pro-inflammatory cytokine since it is produced during the early stages of inflammation and it also activates lymphocytes and has microbicidal activities (Barnes *et al.*, 1998). However, it also has several anti-inflammatory properties. Unlike TNFα and IL-1β, IL-6 does not induce the production of major inflammatory mediators such as prostaglandins and nitric oxide. In contrast, IL-6 inhibits TNFα and IL-1β synthesis, induces production of proteins with anti-inflammatory potential, and enhances glucocorticoid release (Barton, 1997). Therefore, IL-6 may also protect the host cells from potentially destructive inflammatory responses.

After encountering an inflammatory stimulus, the antigen presenting cells *e.g.* macrophages will present part of the phagocytosed matter on its cell surface and this acts as an antigen to a precursor T helper (Th0) lymphocyte. The Th0 cells can be differentiated into Th1 and Th2 subsets that secrete different cytokines (Mosmann and Sad, 1996). The cytokine productions of these different subsets are associated with different kinds of inflammatory responses. Th1 cytokines such as interferon-γ (INFγ), IL-2, and IL-12 are mainly associated with cell-mediated inflammatory reactions whereas Th2 cytokines such as IL-4, IL-5, IL-6, IL-10 and

IL-13 favor antibody production, especially IgE, by B lymphocytes and enhance eosinophil proliferation and its function.

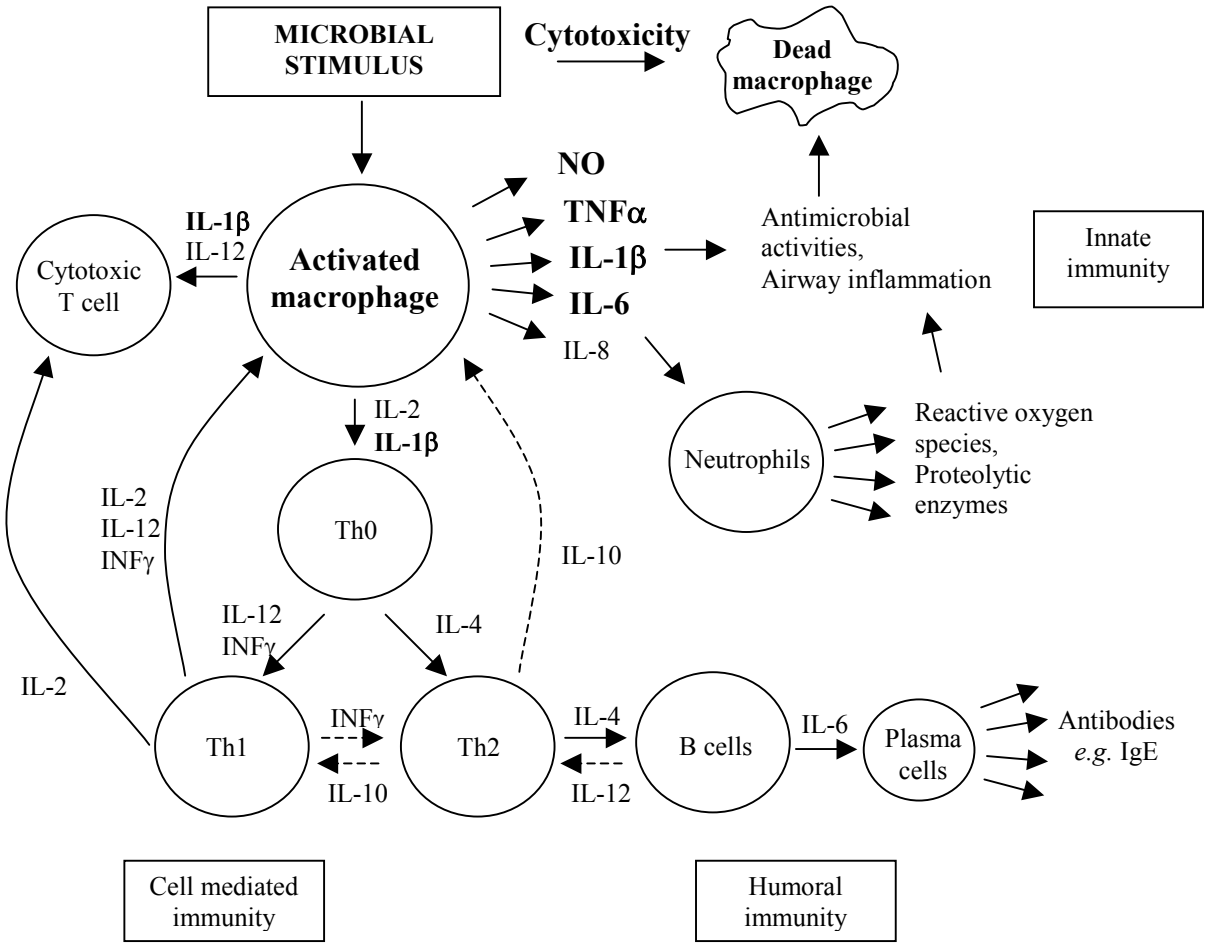

Figure 2.1. Schematic presentation of cytotoxic and inflammatory responses against microbial stimulus in airway macrophages. Parameters studied in this thesis are presented in bold typeface. Solid arrows (→) = activation, dashed arrows (⇢) = inhibition

Innate and Th1-dependent immunities play essential roles in the host defense against microbial infections (Romani, 1997; Cenci *et al.*, 1999; Engele *et al.*, 2002), whereas Th2 cytokines and antibody production by B cells contribute to humoral immunity that is closely related to allergic responses (Mosmann and Sad, 1996; Riccio *et al.*, 2002). In order to achieve a successful and effective immune response, there should be balance between Th1 and Th2 responses.

2.5.1.2 Nitric oxide

Nitric oxide (NO) is an important gaseous mediator of many biological processes including modulation of airway and vascular tone, inflammatory cell activation, neural activation, and mucus secretion (Folkerts *et al.*, 2001; Fischer *et al.*, 2002; Nevin and Broadley, 2002). A variety of cells can produce NO enzymatically from L-arginine by NO synthase (NOS). There are three isoforms of NOS: constitutively expressed neuronal and endothelial NOS (nNOS and eNOS, respectively) and inducible iNOS (Moncada *et al.*, 1991). In contrast to constitutively expressed forms of NOS, iNOS is not normally expressed but during inflammation its synthesis is induced in macrophages by *e.g.* pro-inflammatory cytokines and bacterial endotoxin (LPS) leading to production of high levels of NO for a long period of time (MacMicking *et al.*, 1997).

In airways, NO has both beneficial and deleterious roles. The formation of low amounts of NO in airway endothelial cells by eNOS can be considered as part of the innate host defense against invading organisms. In contrast, the large amount of NO produced in alveolar macrophages and epithelial cells after expression of iNOS in response to a variety of microbial products or inflammatory mediators such as INFγ, TNFα and IL-1β is potentially pro-inflammatory and it may even damage the surrounding cells and tissues (Clancy and Abramson, 1995; MacMicking *et al.*, 1997). However, the ability of different cytokines to elicit the expression of iNOS in rodent and human cells varies. In addition, NO production is usually more pronounced in rodent than in human cells (Clancy and Abramson, 1995, Huttunen *et al.*, 2001). Increased expression of iNOS in inflammatory cells from airways as well as elevated NO levels in exhaled air have been found in many pulmonary diseases including asthma (Hamid *et al.*, 1993; Saleh *et al.*, 1998; Kharitonov and Barnes, 2001). Induction of iNOS in asthma is also likely to be attributable to pro-inflammatory cytokines and other inflammatory mediators (Fischer *et al.*, 2002). At the level of gene expression, transcription factor NF-κB is one of the critical regulators of several inflammatory mediator genes including the genes coding for the synthesis of pro-inflammatory cytokines (Beg and Baltimore, 1996; Fan *et al.*, 2001). NO plays an important role in regulating the release of these mediators by modulating the activation of NF-κB (Thomassen and Kavuru, 2001).

2.5.2 Cytotoxicity

Cytotoxicity is one of the most important mechanisms whereby host cells can initiate non-specific immunity against invading microorganisms (Rothe *et al.*, 1993; MacMicking *et al.*, 1997). The cytotoxic effects of inflammatory mediators such as NO and pro-inflammatory cytokines, especially TNFα, have been reported to play a key role in several pathological conditions secondary to cell damage in various cell types including cortical cells (Bonfoco *et al.*, 1995), osteoblasts (Damoulis and Hauschka, 1997), tumor cells (Davis *et al.*, 2000) and β-cells (Liu *et al.*, 2000). The critical role of TNFα-mediated cytotoxicity in the early phases of the acute respiratory distress syndrome have also been reported (Hamacher *et al.*, 2002). Cell death could be induced by two distinct mechanisms, necrosis or apoptosis. Necrosis usually results from toxic or traumatic events and is characterized by passive cell swelling, destruction of cell organelles and cell lysis that often leads to inflammation. In contrast, apoptosis is an active and controlled process where cells shrink along with chromatin condensation and DNA fragmentation usually without producing any inflammation (Oberholzer *et al.*, 2001). Apoptosis is a normal cellular process that is crucial for tissue remodeling and development. Several of the humoral factors such as TNFα, Fas ligand, and glucocorticoids that initiate and regulate the innate immune response, may also induce apoptosis in lymphocytes and neutrophils (Oberholzer *et al.*, 2001). This suggests that these cell populations are at least partially regulated by apoptosis during inflammation.

Cytotoxicity induced by exposure to compounds present in moisture damaged buildings may be a critical factor behind the adverse health effects. The trichothecene mycotoxins produced by *Stachybotrys* species are known to be extremely toxic to leukocytes and other rapidly dividing cells and they have been shown to induce apoptotic cell death (Yang *et al.*, 2000; Nagase *et al.*, 2002). However, the relative importance of the balance between necrosis and apoptosis during airway inflammation as a result of exposure in moisture damaged buildings remains obscure.

2.6 Assessment of biological activity of microbes grown in buildings

Even though the exposing agents or mechanisms that lead to adverse health effects in the occupants of moisture damaged buildings are not clear, there are indications that both toxic and inflammatory reactions are involved. If we are to evaluate the harmfulness of the moisture damage, it is important to have methods to detect the biological activity of potential exposing agents. There are different bioassays that have been used to study the biological activity of microbial growth in buildings (Table 2.2). Most of them are used to detect the toxicity of microbial species known to produce mycotoxins. To achieve high sensitivity, these assays are usually performed using cell lines other than inflammatory cells. Thus, their relevance in the immunological host defense system remains unclear. However, these methods may have importance in screening for the presence of specific toxins in building samples. Allermann *et al.* (2002) have used the production of IL-8 in human lung epithelial cells as a way for assessing the inflammatory potential of floor dust (Table 2.2). In this kind of biological activity analyses of environmental samples, the actual substances eliciting the detected activity are usually not known.

In this thesis, bioassays were performed using the mouse macrophage cell line that indicated both cytotoxicity and inflammatory potential of microbial spores collected from plasterboard samples. The parameters including cytotoxicity and production of NO as well as pro-inflammatory cytokines TNFα, IL-1β, and IL-6 were selected for this study due to their important role in the innate host defense against invading organisms. In order to verify the biological activity of different microbial species as well as to elucidate the effects of modifications made on plasterboards, the studies were carried out with pure cultures of *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium spinulosum*, or *Streptomyces californicus*.

Table 2.2 Methods that have been used for assessing the biological activity of microbes from moisture damaged buildings.

Response type	Cell type	Detection method	Reference
Toxicity	Fetal feline lung cells	MTT cytotoxicity test	Andersson *et al.* 1997
	Swine kidney cells	MTT cytotoxicity test	Yike *et al.*, 1999
	Mouse macrophages	MTT cytotoxicity test	Hirvonen *et al.*, 1997b
	Mouse hepatoma cells	Inhibition of protein synthesis	Nieminen *et al.*, 2002
	Escherichia coli	DNA repair assay for genotoxicity	Nieminen *et al.*, 2002
	Boar spermatozoa	Inhibition of motility	Andersson *et al.*, 1997 Peltola *et al.*, 2001a, b
	Tracheal rings of chicks	Inhibition of cilia motility	Pieckova and Kunova, 2002
	Rabbit reticulocyte	Inhibition of protein synthesis	Yike *et al.*, 1999
	Sheep blood agar	Hemolytic activity	Vesper *et al.*, 1999
Inflammatory response	Human lung epithelial cells	Production of IL-8	Allermann *et al.*, 2002
	Human lung epithelial cells	Production of TNFα, IL-6 and NO	Huttunen *et al.*, 2001 & 2003
	Mouse macrophages	Production of TNFα, IL-6, NO, or ROS	Hirvonen *et al.*, 1997a, b Huttunen 2003

3 AIMS OF THE STUDY

The overall aim of the study was to investigate the factors that affect microbial growth on plasterboards and the biological activity of spores assessed as induction of cytotoxicity and inflammatory responses *i.e.* the production of nitric oxide and cytokines in mouse macrophages. The specific aims were to study:

1. The differences in the ability of six commercially available plasterboards to support growth of *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium spinulosum*, and *Streptomyces californicus* as well as the biological activity of their spores (Study I).

2. The possible association between the growth patterns of the microbes and the biological activity of the spores (Study II).

3. The role of the main plasterboard components, liners and core, on microbial growth and biological activity of spores (Study III).

4. The role of the core composition on microbial growth and biological activity of spores of *Stachybotrys chartarum* (Study IV) and *Streptomyces californicus* (Study V).

4 MATERIALS AND METHODS

4.1 Plasterboards

In studies I-III, six different commercially available plasterboards were used. In studies IV-V, reference plasterboard and a total of 12 modifications, which were prepared in co-operation with the manufacturers, were investigated. The studied microbes were inoculated on sterilized and wetted liners of the complete plasterboard, except for study III, where microbes were inoculated on liners and cores, separately. The characteristics of the studied plasterboards are presented in Table 4.1, and the modifications made in the plasterboard composition used in studies IV-V are shown in Table 4.2.

Table 4.1. Characteristics of the plasterboards used in studies I-V.

Study	Number of boards	Board type	Composition	Inoculated material	Sterilization of boards
I-II	6	commercial	not available	complete board	170 ^{o}C, 60 min
III	6	commercial	not available	liner and core	170 ^{o}C, 60 min
IV-V	13	modified	available, see Table 4.2	complete board	γ-rays, 25 kGy

4.2 Microbes

Three fungal strains, *Stachybotrys chartarum* HT580 (I-IV), *Aspergillus versicolor* HT486 (I-III) and *Penicillium spinulosum* HT581 (I-III), and a gram-positive bacterium, *Streptomyces californicus* A4 (I-III, V) were used in these studies. *Aspergillus versicolor*, *P. spinulosum*, and *S. californicus* were isolated from indoor air of mould problem buildings by the six-stage impactor (Graseby Andersen, Atlanta, GA, USA). *Stachybotrys chartarum* was isolated from a material sample of a mold-damaged building. All the fungal strains were identified morphologically at the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). *Streptomyces californicus* was identified on the basis of the partial 16S rDNA sequence and physiological tests at the German Collection of Microorganisms and Cell Cultures (DSMZ).

Table 4.2. Composition modifications of the 13 plasterboards used in studies IV-V. The reference board (1) contained a standard amount of natural gypsum, starch and three different additives in the core, and it had a normal paper liner. The changes made in this composition are presented with bold typeface. 1 = presence of the component at the normal concentration. 0 = component removed. Blank = the same composition as in the reference board.

Modified	Board	NG[a]	DSG[b]	Recycled	Starch	Additive 1	Additive 2	Additive 3	Biocide	Paper
Gypsum	1 (ref)	1	0	0	1	1	1	1	0	Normal
	2	**0**	**1**							
	3	**0.9x**		**0.1x**						
	4	**0.5x**		**0.5x**						
Starch	5				**0**					**No Starch[c]**
	6				**0**					
	7				**3x**					
Additives	8					**0**				
	9						**0**			
	10							**0**		
	11				**0**	**0**	**0**	**0**		
Biocide	12								**1**	
	13									**Biocide[d]**

[a] Natural gypsum

[b] Desulforisation gypsum

[c] Normal paper but no starch

[d] Normal paper treated with 1 % Parmetol DF 17 (Schülke & Mayr UK Ltd)

4.3 Growth conditions

Stachybotrys chartarum, *Aspergillus versicolor*, and *Penicillium spinulosum* were cultivated on 2% malt-extract agar (MEA) (Biokar) at 25^{o}C, and *Streptomyces californicus* on tryptone - yeast extract - glucose (TYG) agar (Bacto Plate Count Agar, Difco, Detroit, MI, USA) at 20-23 ^{o}C in the dark until they sporulated. The spores of each strain were collected with a sterile plastic rod into 10 ml of Hanks balanced salt solution (HBSS) (Gibco Lab., Paisley, UK). An amount of 10^6 spores was inoculated on the liners of wetted plasterboard samples (I-II, IV-V) or on the separate liners and cores (III) (Table 1.) Duplicate samples were kept under saturated humidity conditions in sterilized glass vessels at 18-23 ^{o}C. Identically treated plasterboards without inoculations were used as controls. The vessels were aerated with filter-sterilized (0.2 μm, Schleicher & Schuell GmbH, Germany) air once a day for 10 min at a flow rate of 400 ml min^{-1}(Figure 4.1).

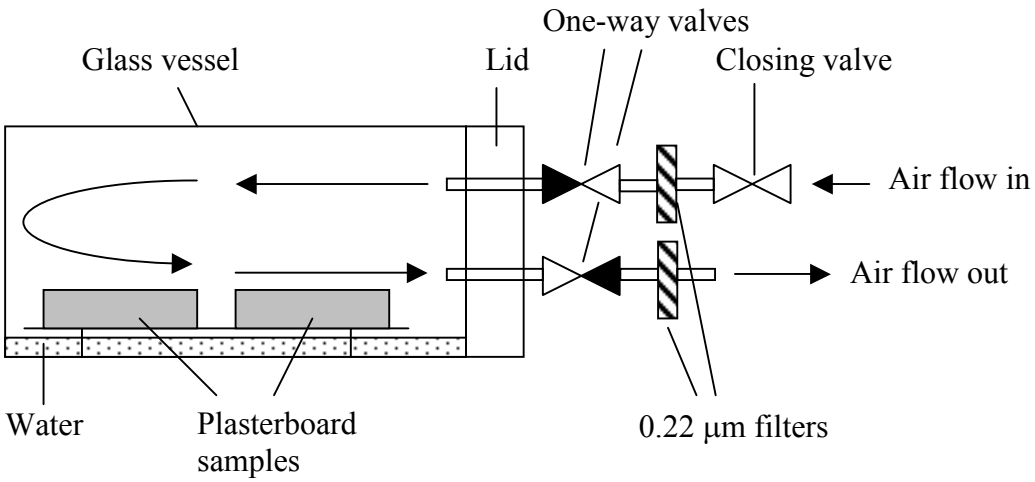

Figure 4.1. Experimental setting of the growth studies

4.4 Biomass analyses

4.4.1 Visual observation (I-III)

The microbial growth and sporulation on plasterboards were observed visually three times a week until no changes had occurred during the last 14 days. The growth was evaluated as the percentage (20, 40, 60, 80 and 100 %) of the maximum amount of biomass achieved for each microbe. From the obtained growth curve, the growth rate was determined as the slope in the exponential growth phase (II).

4.4.2 Ergosterol analysis (IV)

Ergosterol is a component of cell membranes of filamentous fungi which is absent in bacteria and most higher plants. It has been used as a chemical marker for fungal biomass (Schnürer, 1993; Miller and Young, 1997; Saraf *et al.*, 1997; Pasanen *et al.*, 1999). The ergosterol content of *Stachybotrys chartarum* grown on different plasterboards was determined based on the methods published by Axelsson *et al.* (1995) and Pasanen *et al.* (1999). Ergosterol standards (Sigma, St. Louis, Mo., USA) and detached liners were heated with 10% KOH in methanol. 7-Dehydrocholesterol (Sigma, St. Louis, Mo, USA) was used as an internal standard. The samples were purified on a silica gel column. Ergosterol was trimethylsilyl (TMS)-derivatized and analysed with Hewlett-Packard (Palo Alto, California, USA) model

G1800A gas chromatograph equipped with a mass selective detector (GC-MS) and HP7673 automatic sampler.

4.4.3 Fatty acid analysis (V)

Bacterial biomass can be quantified by analyzing the total content of fatty acid methyl esters (FAME). The total content of FAMEs of *Streptomyces californicus* grown on different plasterboards was analyzed according to the method by Suutari *et al*. (1990). Liners were detached from the plasterboards, frozen at -20°C and lyophilized (Edwards 4 K Modulyo freeze dryer, Crawley, England). For the quantification of FAMEs, tridecanoic and nonadecanoic acid methyl esters (Sigma, St. Louis, MO, USA) were added as internal standards. The samples were saponificated, methylated and extracted as methyl esters. FAMEs were analyzed with Hewlett-Packard (Palo Alto, California, USA) model G1800A gas chromatograph equipped with a mass selective detector (GC-MS) using total ion monitoring.

4.5 Total spore numbers (I-V)

The total spore concentration of suspensions of *Stachybotrys chartarum, Aspergillus versicolor*, and *Penicillium spinulosum* was counted using light microscopy and a Bürker counting chamber. The spore density of *Streptomyces californicus* was determined by acridine orange direct counting (AODC) where bacteria were filtered on 0.22 um Nuclepore membrane filter, stained with 0.01 % acridine orange dilution and counted using an epifluorescence microscope (Hobbies *et al*., 1977).

4.6 Biological activity of spores

4.6.1 Macrophage cell culture (I-V)

The mouse macrophage cell line RAW264.7 (American Type Tissue Collection; Rockville, MD, USA) was grown at 37^{o}C, 5 % CO_2, in RPMI Medium 1640 (Gibco Lab., Grand Island, NY,

USA) supplemented with 10 % fetal bovine serum (HyClone Lab., Logan, Utah, USA), 1 % L-glutamine, and 1 % PNS antibiotic mixture (Gibco Lab., Grand Island, NY, USA). The cells were allowed to adhere for 24 h, and then non-adherent cells were washed with fresh complete RPMI medium.

4.6.2 Exposure of the macrophages (I-V)

Fresh complete RPMI was added onto the cells and doses of $1x10^4$, $1x10^5$ or $1x10^6$ spores /10^6 macrophages of *Stachybotrys chartarum, Aspergillus versicolor, Penicillium spinulosum* or *Streptomyces californicus* were added to the cell culture medium. After incubation for 24 h, the cells and the culture medium were collected and centrifuged. The effects of the spores on cell viability, and the nitrite concentration in the cell culture medium were analyzed. The rest of the supernatants and the cells were stored at -80 ^oC for the determination of the cytokines (TNFα, IL-1β, and IL-6) and the Western blot analysis for expression of iNOS.

4.6.3 Nitric Oxide (I-V)

4.6.3.1 Nitrite (I-V)

NO was assayed in the cell culture medium by measuring the stable NO-oxidation product nitrite (NO_2^-) using the method based on the Griess reaction (Green *et al.*, 1982). Briefly, 50-µl aliquots of cell culture medium and standards were incubated in duplicate with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) on a 96-well microtiter plate (Maxisorb, Nunc, Naperville, USA). Chromophore absorbance at 540nm was determined using a microplate reader (iEMS Reader MF, Labsystems, Finland). The nitrite concentration was assessed using sodium nitrite as the standard.

4.6.3.2 Western blot analysis of iNOS (I)

Expression of the NO production catalyzing enzyme, inducible NO-synthase (iNOS), was analyzed with Western blot method. The cells were lysed and the lysates were subjected to electrophoresis through 7.5% SDS-PAGE and proteins were electrophoretically transferred to a nitro-cellulose filter. The filters were treated with iNOS rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, CA) after which an alkaline phosphatase-conjugated second antibody AP-Goat Anti-Rabbit IgG (Biorad, Hercules CA) was added. The filters were developed using BCIP/NBT (330 μg/ml nitro blue tetrazolium/ 165 μg/ml 5-bromo-4-chloro-3-indolyl phosphate disodium).

4.6.4 Cytokine analysis (I-V)

TNFα, IL-6, and IL-1β concentrations were measured from cell culture medium using enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

4.6.5 Cytotoxicity (I-V)

The cytotoxicity of the spores in the cell culture was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,3-dimethyltetrazolium bromide (MTT) test (Sigma, USA) where exogenously administered MTT-solution is converted to the colored formazan in intact mitochondria of the cells, and this product can be photometrically determined (Mosmann, 1983). Briefly, a cell suspension and MTT-solution were added in duplicate to each well in the 96-well plate. After 2 h of incubation, SDS buffer was added. After an overnight incubation, the absorbance at 570 nm was measured with a microplate reader (iEMS Reader MF, Labsystems, Finland). The viability of the cells was calculated in relation to the viability of the control cells.

The microbiological and biological activity parameters measured in studies I-V are summarized in Table 3.

Table 3. Microbiological and biological activity parameters measured in studies I-V. Biomass and sporulation were measured from the surface samples collected from the plasterboards. NO, cytokines and cytotoxicity were analyzed from the cell culture medium of RAW264.7 macrophages after 24-h exposure of microbial spores collected from the plasterboards.

Parameter	Method	Reference	Study
Biomass			
S. chartarum	Visual observation		I-III
	Ergosterol	Axelsson *et al.* 1995	IV
A. versicolor	Visual observation		I-III
P. spinulosum	Visual observation		I-III
Str. californicus	Visual observation		I-III
	Fatty acids	Suutari *et al.* 1990	V
Sporulation	Microscope counting (Bürker)		I-IV
	AODC	Hobbies *et al.* 1977	I-III, V
NO			
Nitrite	Griess	Green *et al.* 1982	I-V
iNOS	Western blot	Hirvonen *et al.* 1996	I
Cytokines			
TNFα,	Enzyme-linked	Jussila *et al.* 1999,	I-V
IL-1β,	immunosorbent assay	Huttunen *et al.* 2001	I-III
IL-6	(ELISA)		I-V
Cytotoxicity	MTT test	Mosmann 1983	I-V

4.7 Composition analyses of cores (IV-V)

Elementary composition of plasterboard core materials was analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES), with Thermo Jarrell Ash Iris/Dual View (Thermo Elemental, USA), after hot (+90 ^{o}C) aqua regia digestion (Niskavaara, 1995). For analyses of nutrient contents and pH of plasterboard cores, 10 g of core material was eluted with 75 ml of ion-exchange-purified water. From this solution, assimilable organic carbon (AOC) was determined according to van der Kooij, *et al.* (1982) and APHA (1992). Total organic carbon (TOC) was analyzed with a high temperature combustion method using a Shimadzu TOC-5000 Analyzer (Kyoto, Japan). The nitrate concentration was determined spectrophotometrically with flow injection analysis according to standard SFS-EN ISO 13395. Ammonium and phosphate concentrations were determined spectrophotometrically according to Lachat QuikChem Methods 10-107-06-1-F and 10-115-01-1-Q (Zellweger Analytics Inc.; Lachat Instruments Divison, Milwaukee, WI, USA). The pH of the solution was measured with Orion Research 611 pH/millivolt meter (Orion Research, Inc., MA, USA).

4.8 Statistical analyses

Statistical analyses of data were performed using the SPSS for Windows program. For comparison of material control and spore-induced inflammatory responses, one-way analysis of variance and Scheffe's multiple comparison test were used for normally distributed data. When data were not normally distributed, Kruskal-Wallis and Dunn's tests (Zar, 1999) were used (I, III). Principal component analysis (PCA) was used to elucidate major variation and covariation patterns for microbial growth, inflammatory response and cytotoxicity data. The correlations between these parameters were studied by the Pearson correlation analysis (II). One-way analysis of variance and Dunnett's test were used for comparing ergosterol (IV) or total fatty acid content (V) and spore contents of sample boards to reference board. The correlation between ergosterol (IV) or total fatty acid (V) content and the amount of spores were studied by the Spearman correlation analysis.

5 RESULTS

5.1 Microbial growth on plasterboards

5.1.1 Effects of plasterboard type on the growth of the four microbial species (II)

The susceptibility of plasterboards for microbial growth was studied by growing *Stachybotrys chartarum*, *Aspergillus versicolor, Penicillium spinulosum* and *Streptomyces californicus* on both sides of six unused, commercially available plasterboards under saturated humidity conditions. The microbial growth, assessed as the duration of the lag time and the amount of biomass produced, did not differ significantly between the front and rear sides of plasterboards. The only major exception was the growth of *S. chartarum* on plasterboard 6, where it was clearly more extensive on the front side than on rear side of the board (II, Table 1). The results based on microbial growth on front side of the complete plasterboard, on pure front liner, and on core are summarized in Table 5.1.

The microbial growth varied greatly between the different plasterboards, being usually best on plasterboards 3 or 4, and poorest on board 2 (Table 5.1). The growth also varied depending

on which microbial species was examined. The length of the lag phase varied from 5 to 34 days. *Stachybotrys chartarum* started to grow consistently faster than the other studied microbes. In addition, *A. versicolor* and *P. spinulosum* grew poorly on board 6, whereas the growth of *S. chartarum* and *Str. californicus* was abundant on that board.

5.1.2 Role of core and liners of plasterboard (III)

The role of the main plasterboard components on microbial growth was studied by growing the same four microbes on the separate liners and cores of the same plasterboards that were used in study II. In general, growth of these microbes was better on the cores than on the liners (Table 5.1, III, Table 1.). Similarly, as in case of complete plasterboards also the cores of plasterboards 3 and 4 were among those substrates that supported the best growth. *Stachybotrys chartarum* and *Str. californicus* grew better on the core than on the liner of plasterboard 6, whereas *A. versicolor* and *P. spinulosum* grew poorly both on liners and core of that board. *Penicillium spinulosum* grew poorly on all liners.

Table 5.1 Growth of *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium spinulosum*, and *Streptomyces californicus* on the front side of the complete plasterboard, pure front liner and core of six plasterboards.

		Lag (d)			Biomass		
Microbe	**Board**	**Complete board**	**Liner**	**Core**	**Complete board**	**Liner**	**Core**
S. chartarum	1	<8	8-24	<8	++	+++	++
	2	<8	8-24	<8	+	+	++
	3	<8	8-24	<8	++	++	+++
	4	8-24	8-24	<8	+	++	+++
	5	<8	nm	<8	++	nm	++
	6	<8	8-24	<8	+++	++	+++
A. versicolor	1	8-24	>24	8-24	++	+++	++
	2	8-24	>24	8-24	++	+++	++
	3	8-24	8-24	8-24	+++	++	+++
	4	8-24	>24	8-24	++	++	+++
	5	8-24	nm	8-24	+	nm	++
	6	8-24	>24	>24	+	+	+
P. spinulosum	1	8-24	>24	>24	+++	+	++
	2	8-24	>24	>24	++	+	++
	3	8-24	>24	8-24	++	+	+++
	4	8-24	>24	8-24	+++	+	++
	5	8-24	nm	>24	+	nm	++
	6	8-24	>24	>24	+	+	+
Str. californicus	1	>24	>24	8-24	+	++	++
	2	>24	>24	8-24	+	++	++
	3	8-24	>24	<8	+	++	++
	4	8-24	>24	<8	+++	++	+++
	5	8-24	nm	8-24	+++	nm	++
	6	8-24	>24	8-24	+++	+	++

+ = low, ++ = moderate, +++ = high biomass production, Lag = time before exponential growth started, nm = not measured

5.2 Biological activity of spores collected from complete plasterboard (I), liners or core (III)

After the growth phase was completed, the biological activity of microbial spores that were collected from complete plasterboards, liners or cores was assessed as their ability to induce the production of inflammatory mediators NO, TNFα, IL-1β and IL-6 as well as to cause cell death in macrophages. There were clear differences in the biological activity of spores from the four microbes tested. Also, for each microbe, the biological activity specifically depended on the type of plasterboard or whether the microbe was grown on separate liner or core of the plasterboard.

In general, the spores of *Streptomyces californicus* and *Aspergillus versicolor* induced the highest cytotoxicity and dose-dependent production of inflammatory mediators whereas the spores of *Penicillium spinulosum* were usually the least biologically active (I, Figures 1-5, III, Figures 1-4). Spores of *Stachybotrys chartarum* also evoked high cytotoxicity especially when collected from cores (III, Figure 4). Spores collected from plasterboard 2 usually induced the highest responses whereas the biological activity of spores from boards 3 or 4 was among the lowest. Spores of all of the tested microbes induced higher TNFα production and cytotoxicity when they were collected from the cores in comparison to the liners (III, Figures 3-4).

The biological activity of spores collected from complete plasterboards, separate liners or cores of six plasterboards is summarized in Figures 5.1-5.4. The biological activity of the spores of each microbe collected only from front side of the complete plasterboard, on pure front liner and on core is shown. In addition, the responses induced only at the highest tested dose ($1x10^6$ spores/$1x10^6$ cells) are presented. The exception is the production of TNFα which is shown with the middle dose ($1x10^5$ spores/$1x10^6$ cells) because the differences in induced responses between the studied boards were most obvious at this dose.

***Stachybotrys chartarum*.** Spores of *S. chartarum* induced the highest TNFα production when collected from cores whereas spores from complete boards or liners induced a similar but lower TNFα response (Figure 5.1). IL-6 production was only induced after the growth on complete plasterboards. Similarly as in TNFα production, the highest cytotoxicity was induced by spores collected from the cores.

Aspergillus versicolor. Spores of *A. versicolor* collected from complete boards or cores induced the highest TNFα production on boards 1, 2, 5, or 6 whereas the spores collected from liners caused the highest TNFα production on boards 3 and 4 (Figure 5.2). Production of IL-6 was mainly triggered by spores from complete boards. Spores collected from core evoked the highest cytotoxicity on boards 1 and 5 whereas the spores from complete boards or liners induced the most severe cytotoxicity on boards 3 and 4. TNFα production induced by spores collected from core ($r = 0.67$, $p < 0.05$) or liner ($r = 0.82$, $p < 0.01$) correlated positively with the cytotoxicity of those spores. The TNFα production triggered by the spores

collected from complete boards correlated with the IL-6 production ($r = 0.81$, $p < 0.01$) but not with the cytotoxicity.

Penicillium spinulosum. Spores of *P. spinulosum* induced the production of TNFα and IL-6 as well as cytotoxicity mainly when collected from complete plasterboards (Figure 5.3). The spores from the cores also triggered TNFα production and this correlated with the TNFα production induced by spores collected from complete plasterboards ($r = 0.75$, $p < 0.01$).

Streptomyces californicus. Among the four tested microbes, spores of *Str. californicus* collected from the complete plasterboards were the most potent inducers of TNF*a* and IL-6 in mouse macrophages (Figure 5.4). Spores from liners or cores also caused high levels of TNFα formation as well as some IL-6 production. The most severe cytotoxicity was evoked by the spores that were collected from the complete plasterboards 1, 2, 5 and 6 or from the cores 3 and 4. *Streptomyces californicus* was the only microbe studied that induced intense NO production when grown on all plasterboards.

Stachybotrys chartarum

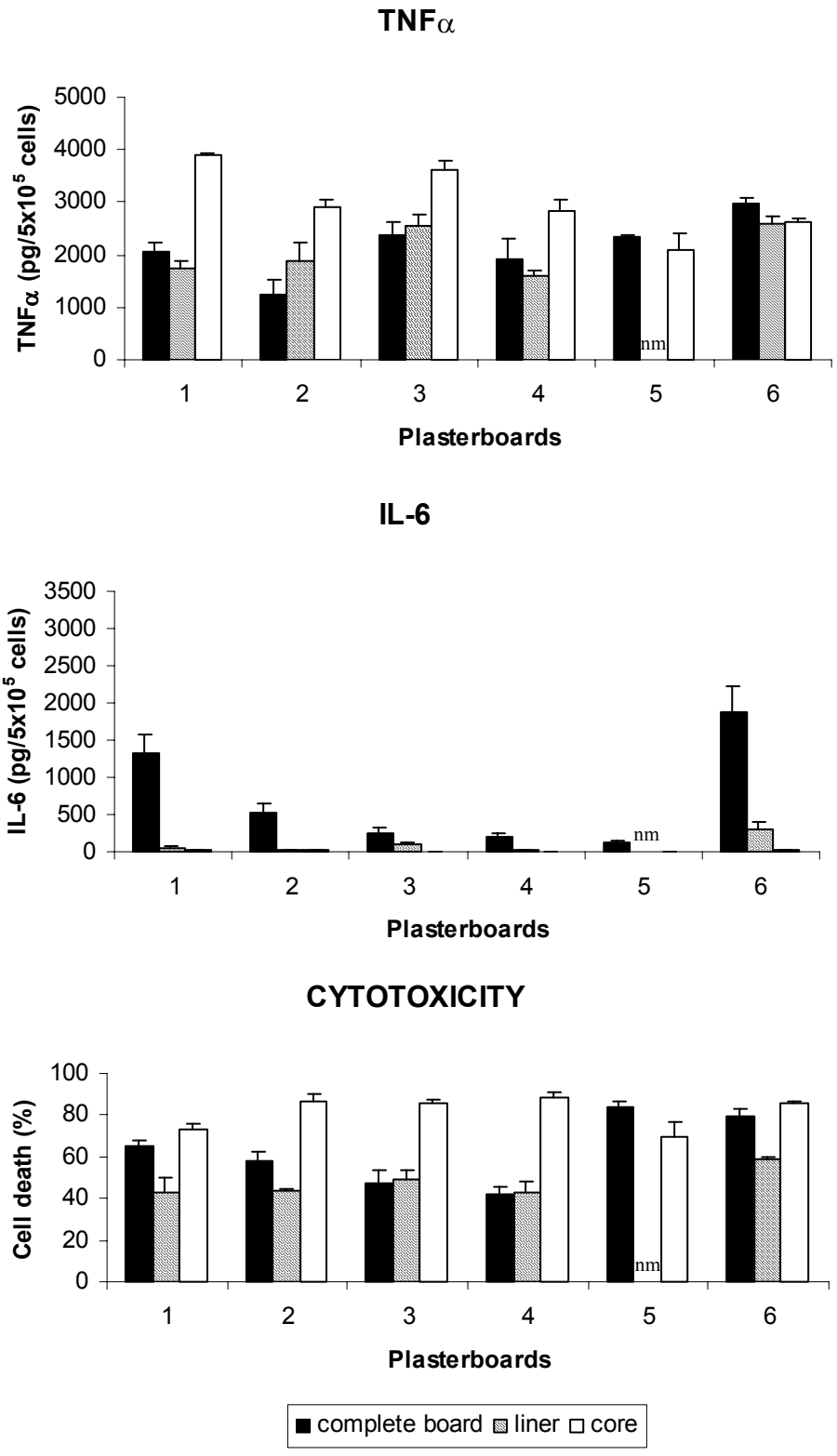

Figure 5.1. Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) production and cytotoxicity induced by spores of *S. chartarum* collected from the front side of complete plasterboard, pure front liner or core of six plasterboards. nm = not measured

Aspergillus versicolor

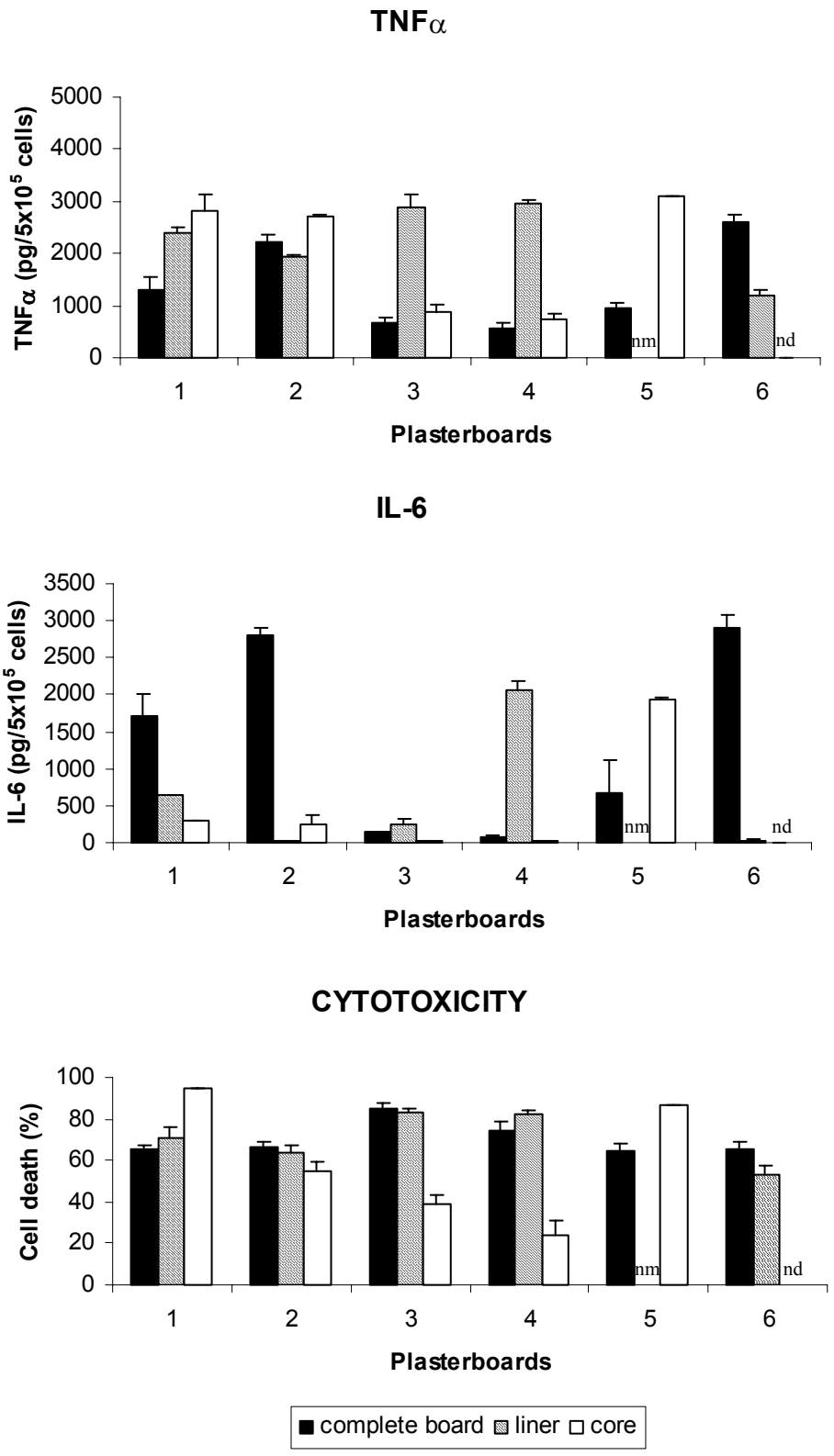

Figure 5.2. Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) production and cytotoxicity induced by spores of *A. versicolor* collected from the front side of complete plasterboard, pure front liner or core of six plasterboards. nd = not detected, nm = not measured

Penicillium spinulosum

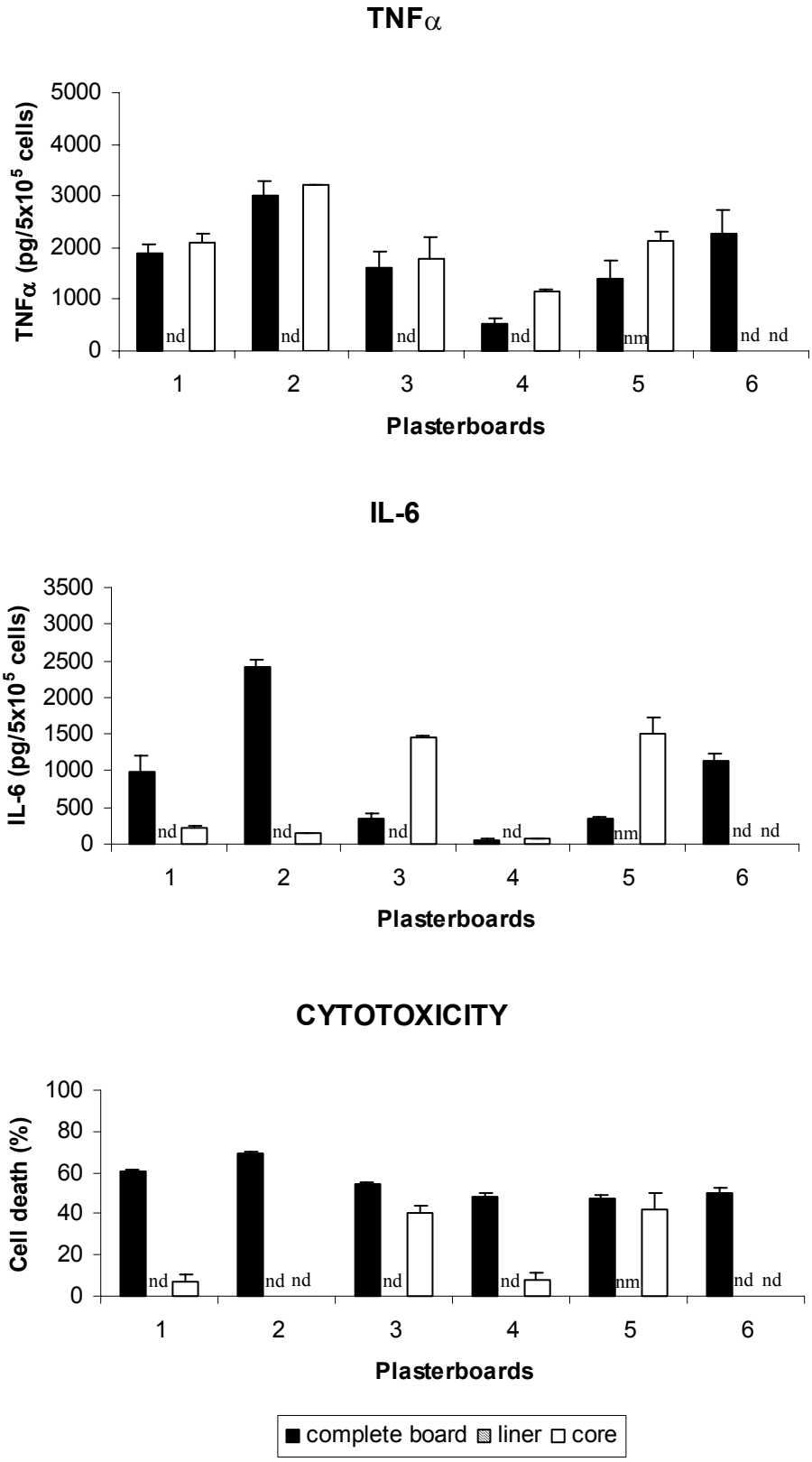

Figure 5.3. Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) production and cytotoxicity induced by spores of *P. spinulosum* collected from the front side of complete plasterboard, pure front liner or core of six plasterboards. nd = not detected, nm = not measured

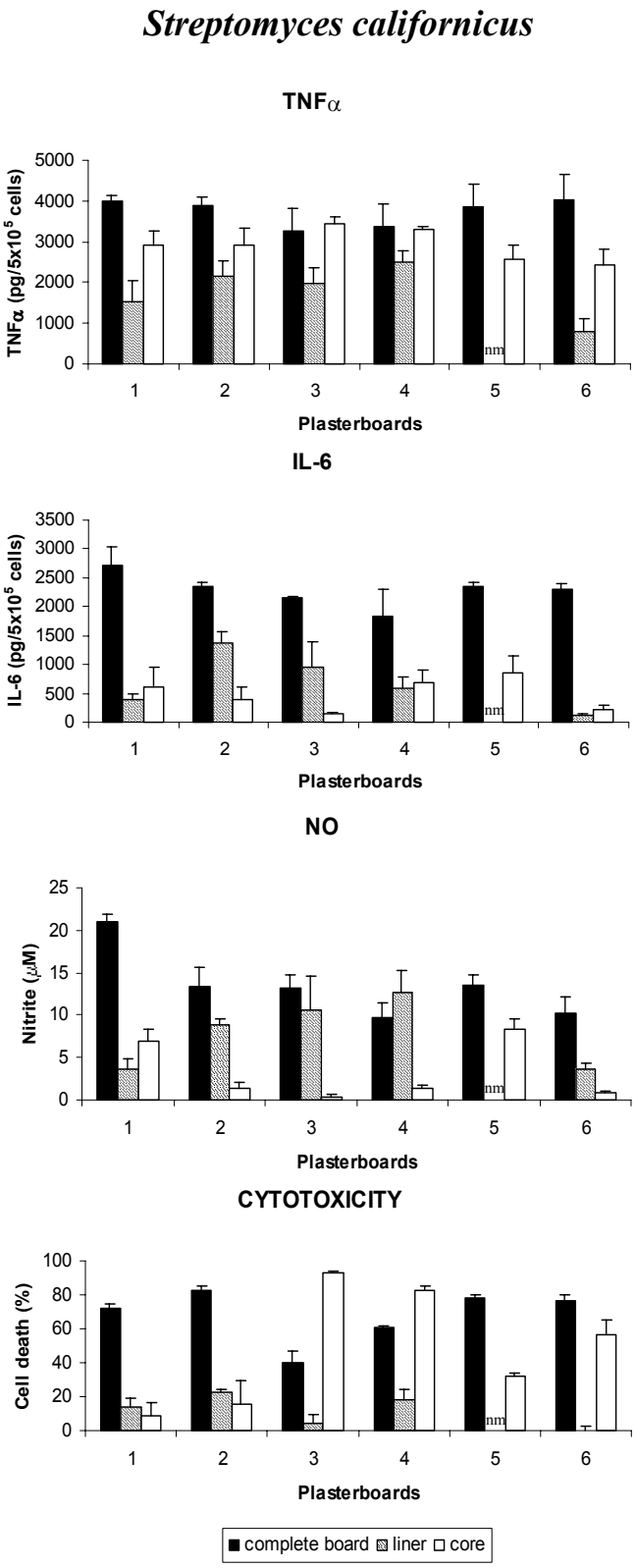

Figure 5.4. Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) production and cytotoxicity induced by spores of *Str. californicus* collected from the front side of complete plasterboard, pure front liner or core of six plasterboards. nm = not measured

5.3 Effect of composition of plasterboard on microbial growth and biological activity of spores (IV-V)

To study further the effects of plasterboard composition on microbial growth and biological activity of spores, *S. chartarum* (IV) and *Str. californicus* (V) were grown under saturated humidity conditions on reference plasterboard and a total of 12 modified plasterboards.

Analyses of plasterboard core composition revealed that the nutrient and trace metal levels of board 2 with desulforisation gypsum (DSG) were considerably lower, excluding arsenic and lead, when compared with the reference board or boards with recycled plasterboards used in the core (IV, Figure 2). In addition, the phosphate concentrations were low in all of the studied plasterboard cores (V, Table 2).

The growth of both *S. chartarum* and *Str. californicus* decreased when compared to reference board in those cases where (a) the liner was treated with biocide, (b) starch was removed from the plasterboard, or (c) DSG was used in the core (Figure 5.5; IV, Figure 1A; V, Figure 1A). The growth of *Str. californicus* also decreased when the starch was removed only from the core or the biocide was added only into the core. On the contrary, addition of biocide only into the core did not reduce S. *chartarum* growth and removal of starch only from the core even increased its growth. Removal of one of the additives from the core slightly increased the growth of *S. chartarum*, whereas the removal of all of the additives from the core decreased the *Str. californicus* growth. Growth of both *S. chartarum* and *Str. californicus* correlated positively with sporulation (IV, Figure 1; V, Figure 1).

The biocide added in the liner (board 13) prevented totally the growth and sporulation of *S. chartarum* and *Str. californicus*. In addition, sporulation of *Str. californicus* was inhibited when the biocide was added into the core. In these cases, no macrophage exposures could be undertaken. Instead, the macrophages were exposed to the spore collection medium from the plasterboard surfaces, but they did not induce any cytotoxic or inflammatory responses. Addition of the biocide into the core did not reduce the growth of *S. chartarum*; in fact the spores collected from that board evoked the highest cytotoxicity. The use of DSG slightly decreased the cytotoxicity of *S. chartarum* (IV, Figure 3A).

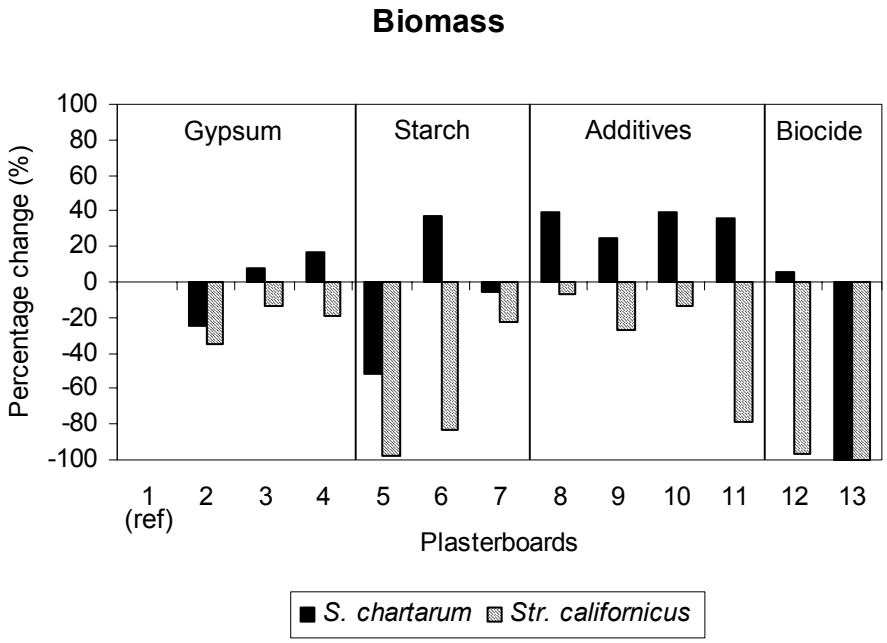

Figure 5.5 Percentage change of biomass of *S. chartarum* and *Str. californicus* grown on modified plasterboards (boards 2-13) compared with the reference board (1). For modifications, see Table 4.2.

Starch had a major impact on growth of both *S. chartarum* and *Str. californicus* but it affected only the biological activity of *Str. californicus* spores. Cytotoxicity and inflammatory responses induced by spores of *Str. californicus* were reduced considerably when the starch was totally removed from the plasterboard (board 5).

The biological activity of the spores of *S. chartarum* and *Str. californicus* collected from the modified plasterboards at the highest tested dose ($1x10^6$ spores/$1x10^6$ cells) is shown in Figure 5.6. In these experiments, spores of *S. chartarum* collected from all of the plasterboards evoked pronounced cytotoxicity and it was at a higher level than that of the spores of *Str. californicus.* On the contrary, spores of *Str. californicus* induced markedly higher inflammatory responses as assessed via production of NO, TNFα, and IL-6 than the spores of *S. chartarum*. In contrast to the commercially available plasterboards used in studies (I-III), the biological activity of *Str. californicus* spores collected from the modified plasterboards correlated positively with the biomass production (V, Figures 1 and 2)

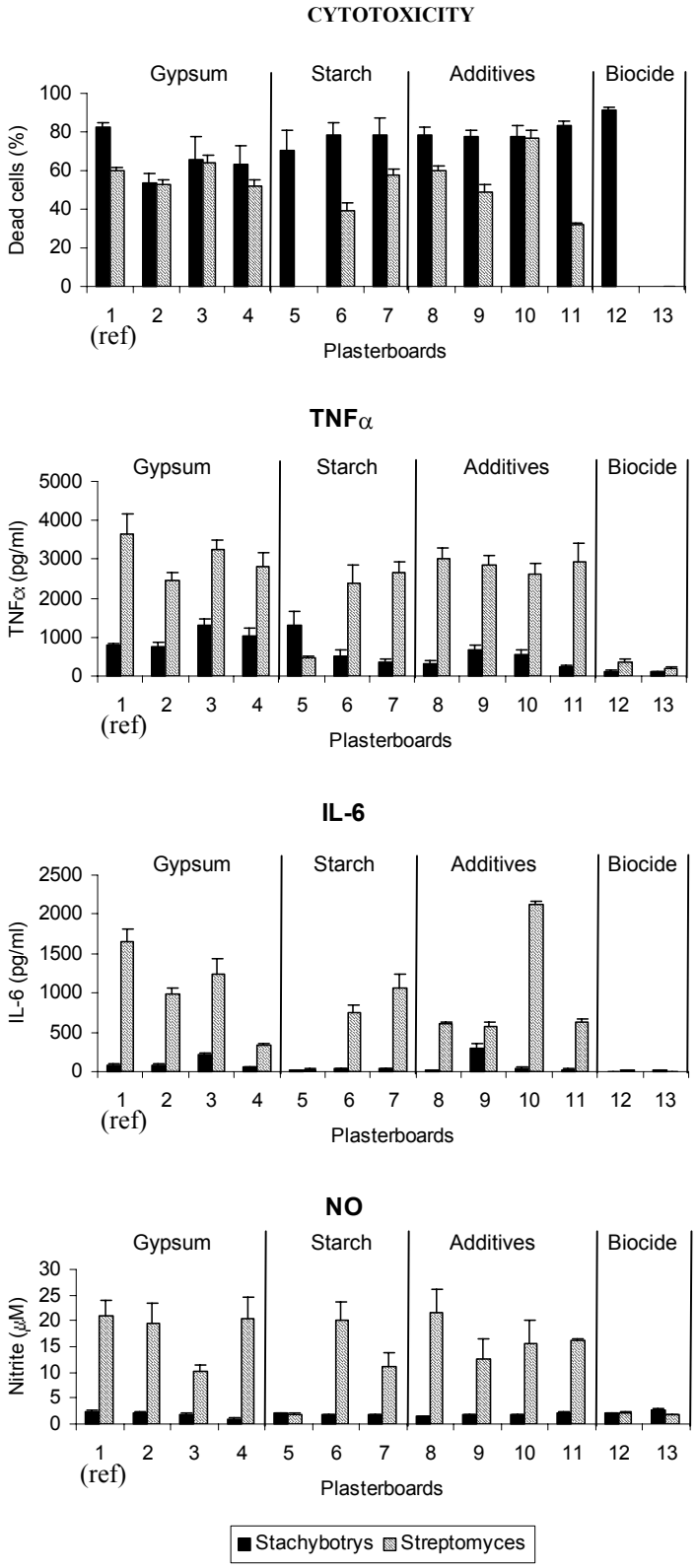

Figure 5.6. Biological activity of spores of *Stachybotrys chartarum* and *Streptomyces californicus* collected from reference plasterboard (board 1) and 12 modified boards. For modifications, see Table 4.2.

6 DISCUSSION

6.1 Effects of plasterboard type on microbial growth and bioactivity of spores

This study revealed that under saturated humidity conditions, *i.e.* conditions simulating severe moisture damage there are major differences between the different types of commercially available plasterboards in their ability to support microbial growth as well as in the subsequent biological activity of the spores. The biological activity of spores was assessed as their ability to induce the production of pro-inflammatory cytokines and nitric oxide in mouse macrophages, which illustrates their potential to induce inflammatory responses in mammalian cells as well as their capabilities to evoke cytotoxicity.

From the six studied commercially available plasterboards, boards 3 or 4 generally supported better growth than board 2. This was seen with all four tested microbes, especially when they were grown on complete plasterboards or on cores. On the other hand, *Aspergillus versicolor* and *Penicillium spinulosum* grew poorly on board 6, whereas *Stachybotrys chartarum* showed quite extensive growth on that board. These observations indicate that microbial growth on plasterboard might be affected by changing the nutritional conditions provided by the plasterboards. Moreover, the biological activity of spores appeared to be influenced by the different growth patterns on plasterboards. Good microbial growth on complete plasterboard was associated with a low bioactivity of the spores, whereas the spores collected from plasterboard supporting only weak growth usually were biologically active. This may be attributable to the formation of different secondary metabolites during the experiment. This interpretation is in line with previous observations that have revealed an inverse correlation between the specific growth rate and the formation of secondary metabolites (von Döhren and Gräfe, 1997).

It has been demonstrated that the growth of the bacterium *Streptomyces anulatus* on concrete, mineral wool, and plasterboard (Roponen *et al.*, 2001) or on different laboratory culture media (Hirvonen *et al.*, 2001) markedly affects the ability of spores to induce inflammatory responses and to evoke cytotoxicity in mouse macrophages. In addition, after growth on defined laboratory culture media, only bacterial spores of *Streptomyces californicus* or

bacterial cells of *Pseudomonas fluorescens* showed significant biological activity whereas no such activity was seen with the fungal spores from *S. chartarum*, *A. versicolor* and *P. spinulosum* (Hirvonen *et al.*, 1997b; Huttunen *et al.*, 2003). In contrast to these previous results, the present study demonstrated that also spores of different fungal species such as *S. chartarum* and *A. versicolor* possess significant cytotoxic and inflammatory potential after growth on wetted plasterboards. These findings also suggest that there is great variability in the abilities of building materials to support the microbial growth and this further impacts on the bioactivity of spores. Moreover, the intensity of the responses is highly dependent on the individual microbial species.

6.2 Growth and biological activity of different microbial species on plasterboards

Stachybotrys chartarum. *S. chartarum* grew markedly faster than the other studied microbes on all studied plasterboards. This confirms the conclusions from other studies that *S. chartarum* is an important contaminant of wetted plasterboards (Gravesen et al., 1994; Hyvärinen *et al.*, 2002). The growth of *S. chartarum* in buildings has attracted great attention since it is claimed to be associated with the adverse health effects experienced by the occupants of mold problem buildings (Croft, 1986; Hodgson *et al.*, 1998; Dearborn *et al.*, 1999; Johanning *et al.*, 1999). However, the causality of these effects is not clear. The observations that good growth of *S. chartarum* on plasterboards was associated with intense cytotoxicity of its spores, and the spores of *S. chartarum* were relatively toxic in all the experiments confirms the general view of the toxic potential of this fungus. The reason why *S. chartarum* is thought to be hazardous in indoor environments is the ability of this fungus to produce several highly toxic mycotoxins such as trichothecenes (Gravesen *et al.*, 1999; Nielsen *et al.*, 1998 a,b; Nikulin *et al.*, 1994; Sorenson, 1999). The toxins may be carried along with spores (Sorenson *et al.*, 1987; Pasanen *et al.*, 1993; Nikulin *et al.*, 1996, 1997; Rand *et al*., 2002) and thus inhaled if the spores are liberated into the air.

Interestingly, the growth of *Stachybotrys chartarum* was better on cores than on liners. This is an important finding, since the growth of *S. chartarum* with cellulolytic activity is commonly claimed to occur predominantly on cellulose rich liners of plasterboards (Gravesen *et al.*, 1994; Nikulin *et al.*, 1994; Andersson *et al.*, 1997). Spores of *S. chartarum* collected from

plasterboard cores also induced higher TNFα production and cytotoxicity than the spores collected from liners. These results indicate that the critical factors for *S. chartarum* growth as well as production of biologically active compounds leading to cytoxicity in mammalian cells seem to be located in the plasterboard cores.

The production of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 is generally increased in macrophages after microbial exposure (Cluzel and Lee, 1992). NO is also formed in macrophages after expression of inducible NO synthase (iNOS) in response to immunological or inflammatory stimuli (Moncada and Higgs, 1995). On the plasterboards the spores of *S. chartarum* were cytotoxic but they did not induce any major production of these inflammatory mediators, except for TNFα. This may be due to potent toxins produced by *S. chartarum* while growing on plasterboard, which may lead to such severe cytotoxicity in the macrophages that the cells are killed before they can mount an inflammatory response. This is in concordance with the previous study where highly toxic *Stachybotrys* spp. isolates producing macrocyclic trichothecenes did not induce the production of inflammatory mediators in macrophages whereas the less toxic isolates producing atranones did induce strong inflammatory responses (Nielsen *et al.*, 2001).

Streptomyces californicus. Although several epidemiological studies have highlighted the association between microbial growth and adverse health effects, research has mainly been focused on the detection of molds as the causative agents (Verhoeff and Burge, 1997; Peat *et al.*, 1998). However, there are indications that also bacteria may contribute to the exposure and hence, possibly to the health effects (Nevalainen *et al.*, 1991; Andersson *et al.*, 1997; Peltola *et al*., 2001a,b). In the present study, spores of the gram-positive bacterium, *Streptomyces californicus* induced major inflammatory responses in mouse macrophages including the production of TNFα, IL-1β and IL-6. *Str. californicus* was also the only microbe studied that after growth on all plasterboards induced the expression of iNOS, resulting in intense production of NO. This suggests that *Str. californicus* possesses a major potential to initiate inflammation in mammalian cells after growth on plasterboards. This bacterium also evoked severe cytotoxicity, especially when it was grown on commercially available plasterboards. The biological activity of *Str. californicus* was not surprising, since streptomycetes are well known for their ability to produce biologically active compounds

such as antibiotics, vitamins, and enzymes which are also widely used in industrial fermentation processes (Kutzner, 1986). Several other bacterial species isolated from moisture damaged buildings such as *Mycobacterium* spp., and *Streptomyces anulatus* have also been shown to have toxic and inflammatory potential both in mouse and human cell lines *in vitro* (Hirvonen *et al.*, 1997a; Huttunen *et al.*, 2001) and *in vivo* (Jussila *et al.*, 2001 & 2002b). The species of *Bacillus*, *Nocardiopsis*, and *Streptomyces*, isolated from moisture damaged buildings have also been shown to have toxic effects on boar spermatozoa (Peltola *et al.*, 2001a,b). Based on these findings, it is important also to remember that many bacterial species are found from contaminated material or indoor air in moisture damaged buildings. These microorganisms should not be overlooked when exposing agents and their health effects are to be evaluated.

Aspergillus versicolo*r*. *A. versicolor* grew relatively well on complete plasterboards as well as on liners and core, separately. *A. versicolor* is a frequently found fungal species in moisture damaged buildings (Gravesen *et al*., 1999; Hyvärinen *et al*., 2002), also often associated with adverse health effects among the occupants (Ezeonu *et al.*, 1994; Hodgson *et al*., 1998; Johanning *et al*., 1999). *A. versicolor* has been reported to produce mycotoxins when grown on different building materials including wetted plasterboard. This would at least partly explain the high potential of *A. versicolor* spores to induce inflammatory responses and cytotoxicity in mouse macrophages.

Interestingly, growth of *A. versicolor* was strongly restricted on plasterboard 6, both on complete board and on liners and core, separately. This suggests that the growth of *A. versicolor* could well be prevented if even rather minor changes were made to the nutritional conditions of the plasterboard. However, the spores that were collected from complete board 6 where there was slow growth, did induce among the most intence inflammatory responses. This indicates that with these nutritional conditions, *A. versicolor* may undergo changes in its metabolism and start to produce biologically active secondary metabolites.

***Penicillium spinulosum*.** The relatively poor growth of *P. spinulosum* on plasterboards was surprising since *Penicillium* species are typically regarded as rapid growers and they are most frequently found colonizers on plasterboards in moisture damaged buildings (Hyvärinen *et al.*, 2002). On the other hand, *Penicillium spinulosum* is regarded as a xerophilic primary

colonizer preferring $a_w < 0.8$ (Grant *et al.*, 1989). This would give it a competitive advantage against secondary and tertiary colonizers on gradually moistening building materials or at the less moistened edges of the moisture damage. However, in this study, the plasterboards were wetted and kept under saturated humidity conditions ($a_w = 1.0$). Therefore the moisture conditions on plasterboards did not favor the xerophilic *Penicillium spinulosum* but enabled the growth of those colonizers favoring higher water activity. The reason for the poor growth is more likely due to the deprivation of available nutrients. *P. spinulosum* did not grow on pure liner, only on the core or on complete plasterboard. This suggests that when it grows on surface of complete plasterboard, it utilizes nutrients absorbed from the core. Spores of *P. spinulosum* showed the lowest biological activity among the studied microbes. This emphasizes that the observed inflammatory responses are species specific and are not invariably induced by all microbes present in moisture damaged buildings.

In this study, the experiments were carried out with one single microbial strain at a time under stable environmental conditions. However, in real moisture damage situations, the environmental conditions as well as the dominant microbial species will vary leading to a diversity of microbes and a succession of different microbial populations. It is possible that when competing microbes are growing on the same substrate, this can influence their tendency to produce toxic secondary metabolites against other organisms (Marin *et al*., 1998, Picco *et al*., 1999). Also, exposure to various microbes or their metabolites concurrently may have synergistic inflammatory effects (Norn, 1993; Fogelmark *et al*., 1994). Currently, very little is known about the possible harmfulness of these interactions.

6.3 Effect of composition of plasterboard on microbial growth and biological activity of spores

The studies made with commercially available plasterboards showed that there are major variations between different types of plasterboards in their ability to support microbial growth as well as on the subsequent biological activity of spores. Moreover, the composition of the cores seemed to play a crucial role in these events. Therefore, the effects of core composition on microbial growth and biological activity of spores were studied with reference plasterboard and a total of 12 modifications.

In this study, starch proved to be one of the most important compounds present in plasterboard that could affect microbial growth. Most of the fungi and several *Streptomyces* species are capable of utilizing starch as a carbon source, hydrolyzing it with starch-hydrolyzing enzymes, amylases (Doull and Vining, 1990; Dix and Webster, 1995; He *et al.*, 1995; Raytapadar and Paul, 2001). Starch is a component of paper liners and it is also used for joining the liners to the core. Removal of starch completely from plasterboard, *i.e.* both from the core and the liner, reduced the growth of both studied microbes *Stachybotrys chartarum* and *Streptomyces californicus*. The growth of *Str. californicus* was also reduced when the starch was removed only from core while the growth of *S. chartarum* was not. This indicates that in addition to cellulose (Gravesen *et al.*, 1994; Nikulin *et al.*, 1994; Andersson *et al.*, 1997), also the starch in the liner is an important nutrient source, promoting *S. chartarum* growth. In addition, *Str. californicus* seems to be able to effectively utilize starch also from the core. Total removal of starch attenuated the biological activity of spores of both microbes. However, it did not reduce the cytotoxicity of *S. chartarum*, which emphasizes the toxic potential of this fungus.

Another nutritional factor affecting microbial growth on plasterboard is the gypsum bulk used in the core. Concentrations of carbon and nitrogen (milligrams per liter) and phosphorus (micrograms per liter) extracted from the plasterboard core were low compared to the defined culture media used for cultivation of species of *Stachybotrys* or *Streptomyces*, which typically contain these essential nutrients in the order of grams per liter (Lounes *et al.*, 1996; Samson *et al.*, 1996; Brabban and Edwards, 1997; Karandikar *et al.*, 1997). However, the nutritional composition of different core materials varied depending on the source of the gypsum. This was demonstrated when the amounts of nutrients in the cores decreased along with the increased amount of recycled plasterboard used. Also, the nutrient contents of desulforisation gypsum (DSG) were markedly decreased in comparison with natural gypsum (NG). The growth of both *S. chartarum* and *Str. californicus* was weaker on the board with DSG in comparison to reference board. The nutrient content, surface roughness and average porosity of plain gypsum substrates have been shown to markedly affect microbial growth (Adan, 1994). It has also been reported that phosphogypsum, which is a residue of the phosphoric acid produced from apatite, is much more susceptible to fungal growth compared to natural gypsum (Shirakawa *et al.*, 2002). On the basis of these results it seems that the restricted

growth of *S. chartarum* and *Str. californicus* on DSG board may be at least partially due to its low nutrient content. In addition to the nutrients, also the amounts of certain inorganic ions such as Al^{3+}, K^{+}, Mg^{2+}, and Mn^{2+}, were systematically reduced in DSG board in comparison with reference board. However, in contrast to the nutrients, the quantities of these ions were still so high that they cannot be considered to limit the growth.

The growth of *Streptomyces californicus* on all boards was very slow. It took up to four months for *Str. californicus* to grow and sporulate. It is probable that the delayed growth may have been a response of the organism to the nutrient limitation of the plasterboard cores. In particular, the amount of phosphate in the cores was low. Phosphate is one of the key nutrients required for microbial growth and it has been demonstrated to participate in the regulation of morphological and physiological differentiation. Reduction in the growth rate resulting from phosphate limitation may be the critical factor for the onset of secondary metabolism in streptomycetes (Doull and Vining, 1990; Parro *et al.*, 1998) or sporulation (Kendrick and Ensign, 1983). This would partly explain the intense biological activity of spores of *Str. californicus* after growth on plasterboards.

Conventional additives used in the plasterboard core did not have any major effects on microbial growth or biological activity of spores. Removal of additives one at a time did not affect the growth of *Str. californicus* whereas the growth of *S. chartarum* was slightly increased. This suggests that these single chemicals used in the core to improve the technical characteristics of plasterboard do not contain substrates for growth of these two microbes. They may only have some inhibitory effects on growth of *S. chartarum*. The poor growth and sporulation of *Str. californicus* on board 11, which did not contain any additives, was probably due to the lack of starch rather than the absence of other additives.

In addition to changes in the nutritional composition of the core, the addition of a biocide into the core or on the liner was tested to see if it would have any effect on microbial growth or on the subsequent biological activity of spores. There are several different preservatives and biocides with different inhibition activity that have been used for prevention of microbial growth on gypsum containing building materials (Price and Ahearn, 1999; Shirakawa *et al.*, 2002). In the present study, when the liner was treated with a biocide (1-% Parmetol DF 17), the growths of both *Stachybotrys chartarum* and *Streptomyces californicus* were totally

inhibited. Interestingly, if the biocide was added only into the core, the growth of *Str. californicus* was prevented but not the growth of *S. chartarum*. In addition, the spores of *S. chartarum* collected from that board evoked the highest cytotoxicity. This finding highlights a serious issue. It demonstrates the varying susceptibility of different microorganisms against biocides. If the biocide fails to prevent microbial growth totally, it may in fact cause a major elevation in the biological activity of the residual organisms. Moreover, microbes may gradually become resistant to most biocides over long periods of time. This may contribute to changes in microbial population that could have other still unknown consequences (Russel, 2002). It has also been suggested that the widespread use of biocides in consumer products may favor the development of antibiotic resistance in clinical practice (Levy, 1998; Russel *et al.*, 1999). In summary, if it is decided to include biocides as additives in plasterboards, their effective concentration as well as the persistence of the used chemical should be tested in order to guarantee appropriate antimicrobial activity.

6.4 Practical implications

This study is part of a major program to identify the causal relationships between the exposing agents emitted from the moisture-damaged material and the observed health effects among the occupants. The results show that under conditions of excess moisture, plasterboard contaminated with microbes is a possible source of the agents inducing inflammatory or toxic reactions in mouse macrophages *in vitro*. Similar responses have also been obtained from studies with human cell lines *in vitro* (Huttunen *et al*., 2001) and in mouse models *in vivo* (Jussila *et al*., 2002a,b) after growth of these microbes on laboratory culture media. Similar reactions have also been shown indirectly to occur in humans (Hirvonen *et al*., 1999; Purokivi *et al*., 2001). Altogether, these observations suggest that the inflammatory response against microbial spores could be one of the contributing factors in the etiology of the respiratory symptoms experienced by the occupants of moisture damaged buildings.

This study provides novel information about the complex microbiological behavior of plasterboard that can be used for the development of products that are not so easily attacked with microbes that possess hazardous biological potential. It is not realistic to think that we will create building materials that are totally resistant to microbial growth. However, if the

onset of microbial growth on building material after sudden moisture damage could be delayed, it should be possible to repair the damage before any severe microbial growth has occurred and in this way prevent potential adverse health effects. In modern buildings, several different types of building materials are used, often as composite structures. Therefore, not only the individual building material but also the whole composite structure should be taken into account in any efforts to avoid moisture problems. Ultimately, it seems that the most cost effective and easiest way to avoid moisture related health problems would be the prevention of the moisture damages. This will require improved building design and construction as well as regular and systematic maintenance.

7 CONCLUSIONS

The conclusions that can be drawn from the present study are as follows:

1. There were considerable variations between different commercially available plasterboards in their ability to support microbial growth of the four studied microbes and to cause changes in the induction of inflammatory and toxic reactions in mammalian cells. This suggests that these microbiological and immunological reactions might be affected by changing the nutritional conditions of plasterboards.

2. In general, good microbial growth on plasterboard was associated with a low bioactivity of the spores, whereas the spores collected from plasterboard supporting only weak growth usually are biologically active. However, there were also exceptions from this general pattern.

3. Microbial growth on plasterboards and the subsequent biological activity of spores was not only due to the paper liner of plasterboard, but the core material also had a crucial role.

4. Microbial growth on plasterboard and subsequent biological activity of spores could be affected by minor changes to the composition of core or liners but it could not be totally prevented without resorting to the use of biocides. However, incomplete prevention of microbial growth by biocides may even increase the cytotoxic potential of the spores.

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