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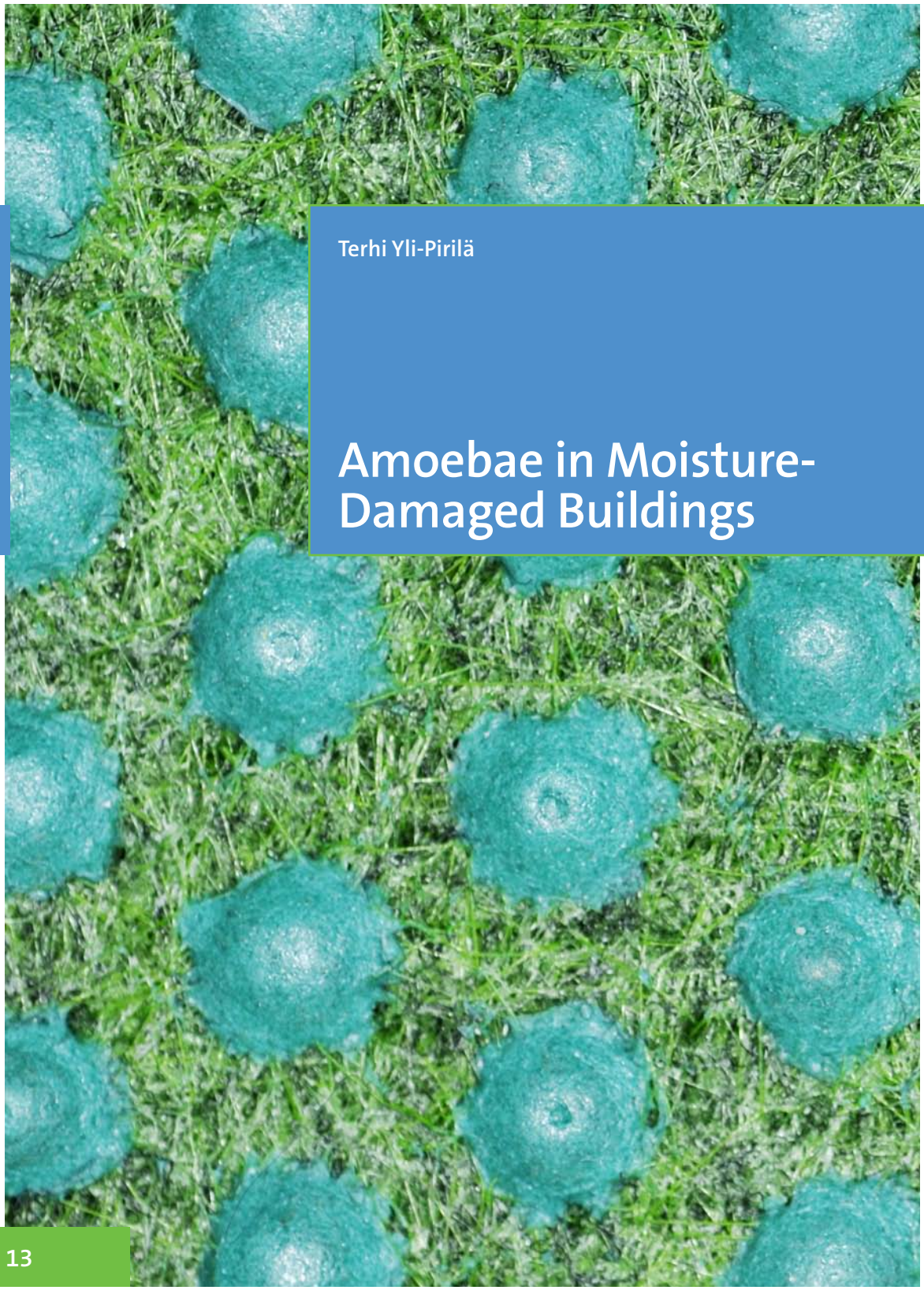

RESEARCH

Terhi Yli-Pirilä

Amoebae in Moisture-Damaged Buildings

Terhi Yli-Pirilä

AMOEBAE IN MOISTURE-DAMAGED BUILDINGS

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Natural and Environmental Sciences, University of Kuopio, for public examination in ML3 auditorium, Medistudia Building, on 8th May 2009, at 12 o'clock noon.

National Institute for Health and Welfare, Helsinki, Finland

and

Faculty of Environmental Sciences, University of Kuopio, Finland

Kuopio 2009

Publications of the National Institute for Health and Welfare THL. Research 13.

ISBN 978-952-245-075-3 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-245-076-0 (pdf)
ISSN 1798-0062 (pdf)

Kannen kuva - cover graphic:

Gummerus printing
Jyväskylä, Finland 2009

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Terhi Yli-Pirilä, Amoebae in Moisture-Damaged Buildings
National Institute for Health and Welfare, Research 13. 99 Pages. Helsinki, Finland 2009. ISBN 978-952-245-075-2 (printed), 978-952-245-076-0 (pdf)

ABSTRACT

Moisture damage in buildings and consequent microbial growth are associated with adverse health effects suffered by the occupants. Although the association is well documented epidemiologically, the exact causative agents for the health effects are not usually known. Even though the microbial network growing on moisture-damaged building materials is a complex ecosystem consisting of many types of organisms including bacteria, yeasts, fungi, protozoa, and mites, exposure has mainly been described in terms of fungal and bacterial diversity and quantity. It is important not to overlook the other possible organisms growing on these materials to better understand the link between the exposure and the symptoms. In this thesis, the occurrence and role of amoebae in moisture damage is elucidated.

First, the prevalence of amoebae in moisture-damaged buildings was estimated by screening 124 building material samples. Then amoebal survival on moist building materials was studied by inoculating samples of building materials with *Acanthamoeba polyphaga* and incubating those in 100 % relative humidity for 0-56 days. Thirdly, the effects of amoebae on other microbes commonly found in moisture-damaged buildings were assessed by co-cultivating three bacterial (*Streptomyces californicus*, *Bacillus cereus*, and *Pseudomonas fluorescens*) and three fungal strains (*Stachybotrys chartarum*, *Aspergillus versicolor*, and *Penicillium spinulosum*) together with *A. polyphaga* and also individually for up to 28 days. Their growth was measured at different times during the incubation. Finally, the effects of this co-culture on the cytotoxic and proinflammatory potential of the microbes were studied by exposing RAW264.7 mouse macrophages to graded doses of co-cultured and individually grown fungi, bacteria and amoebae.

Amoebae were found in 22 % of the samples and they often were detected at the same locations as “indicator microbes” of moisture damage, e.g. with the bacterium *Streptomyces*, and with the fungi *Acremonium*, *Trichoderma*, *Chaetomium*, and *Aspergillus versicolor*. In the inoculation tests, *A. polyphaga* amoebae survived throughout the two-month experiment on samples of mineral insulation, old pine plank, birch plank and gypsum board, often even without nutrient supplementation. All materials with the exception of fresh pine plank, supported amoebal survival at least temporarily. Furthermore, co-cultivation with amoebae significantly increased the growth of all bacteria studied, whereas with fungi, only a modest increase in the

growth was observed. Co-culturing also affected the toxicity and proinflammatory potential of two of the studied strains: the ability of *P. spinulosum* and *S. californicus* to induce the production of inflammatory mediators - nitric oxide, TNFα and IL-6 - in RAW264.7 macrophages was increased manifold. In addition, their cytotoxicity was somewhat increased after incubation with amoebae.

The results of this study show that amoebae are members of the microbial network present in moisture-damaged building materials. The interaction with amoebae could lead to alterations in the properties of the other microbes present in the water-damaged structures. Amoebae may increase the growth of other microbes present, and render the microbes more cytotoxic. Thus, amoebae may indirectly modify the health effects associated with moisture-damaged buildings. However, more evidence from both empirical and epidemiological studies is needed before the role of amoebae as exposing agents in moisture-damaged buildings is fully understood.

Keywords: Amoebae, *Acanthamoeba polyphaga*, bacteria, fungi, moisture damage, buildings, building materials, co-culture, cytokines, NO

Terhi Yli-Pirilä. Amoebae in Moisture-Damaged Buildings. Terveyden ja hyvinvoinnin laitos, Research 13. Helsinki 2009. 99 sivua. ISBN 978-952-245-075-2 (painettu), 978-952-245-076-0 (pdf)

TIIVISTELMÄ

Rakennusten kosteusvauriot ja mikrobikasvusto ovat yhteydessä rakennusten käyttäjien kokemiin terveyshaittoihin. Vaikka tämä yhteys on osoitettu epidemiologisesti, tarkkoja terveysvaikutuksien aiheuttajia ei tunneta. Kosteusvaurioissa esiintyvä mikrobikasvusto on monimutkainen ekologinen kokonaisuus, jossa on mukana bakteereita, homesieniä, hiivoja, alkueläimiä ja punkkeja. Altistusta kuvataan tavallisesti mittaamalla homesienten ja joskus bakteereiden lajikirjoa ja pitoisuutta. Jotta terveysvaikutuksen ja kosteusvaurioituneessa rakennuksessa esiintyvän altistuksen välistä yhteyttä voitaisiin selventää, on tärkeää tutkia myös muita kosteusvaurioissa esiintyviä eliöitä. Tässä väitöskirjatyössä tutkittiin ameboiden esiintymistä ja vaikutuksia kosteus- ja homevaurioituneissa materiaaleissa.

Ameboiden yleisyyttä kosteusvaurioituneissa rakennuksissa selvitettiin viljelemällä amebat 124 rakennusmateriaalinäytteestä. Amebojen selviytymistä eri rakennusmateriaaleilla seurattiin ymppäämällä *Acanthamoeba polyphaga* -amebaa rakennusmateriaalinäytteisiin ja inkuboimalla näitä 100% suhteellisessa kosteudessa 0-56 vrk ajan. Tutkimuksen kolmannessa osassa mitattiin amebojen vaikutuksia muihin kosteusvauriomikrobeihin kasvattamalla kosteusvauriorakennuksista eristettyjä kolmea bakteerikantaa (*Streptomyces californicus*, *Bacillus cereus* ja *Pseudomonas fluorescens*) ja kolmea homesienikantaa (*Stachybotrys chartarum*, *Aspergillus versicolor*, ja *Penicillium spinulosum*) erikseen ja yhdessä *A. polyphaga* -amebakannan kanssa 0-28 vrk ajan. Homesienten, bakteerien ja amebojen kokonaispitoisuudet ja elinkykyisten itiöiden/solujen pitoisuudet määritettiin useissa aikapisteissä inkuboinnin aikana. Lopuksi selvitettiin myös amebojen vaikutuksia näiden kantojen toksisuuteen ja kykyyn aiheuttaa tulehdusvasteita altistamalla hiiren makrofageja (RAW264.7) erisuuruisille annoksille ko. mikrobien itiöitä/soluja.

Ameboja löydettiin 22 % kosteusvaurioituneista rakennuksista otetuista näytteistä, ja ne esiintyivät usein yhdessä kosteusvaurioiden ”indikaattorimikrobien" kanssa, kuten *Streptomyces*-bakteereiden ja *Acremonium*, *Trichoderma*, *Chaetomium*, ja *Aspergillus versicolor* -homesienten kanssa. Kasvatuskokeissa havaittiin, että *A. polyphaga* selvisi elinkykyisenä koko kahden kuukauden inkuboinnin ajan mineraalivillalla, harmaantuneella mäntylankulla, koivulankulla ja kipsilevyllä jopa ilman lisättyä ravintoa. Amebat selvisivät myös muilla materiaaleilla tuoretta mäntyä lukuun ottamatta ainakin hetkellisesti. Ameban vaikutuksia muihin

mikrobeihin selvitettäessä havaittiin, että yhteiskasvatus ameban kanssa lisäsi merkitsevästi kaikkien bakteereiden kasvua ja elinkykyä. Homesienille vaikutus oli vähäisempi. Yhteiskasvatus myös lisäsi kahden tutkitun mikrobikannan toksisuutta ja kykyä aiheuttaa tulehdusvasteita; *P. spinulosum* -homesienen ja *S. californicus* -bakteerin kyky indusoida tulehdusvälittäjäaineiden (typpioksidi, TNF-α ja IL-6) tuotantoa RAW264.7 -makrofageissa moninkertaistui. Myös sytotoksisuus lisääntyi jonkin verran.

Tämä tutkimus osoittaa, että amebat ovat osa kosteusvaurioituneissa rakennuksissa esiintyvää mikrobiverkostoa. Vuorovaikutus amebojen kanssa voi muuntaa toisten kosteusvaurioissa kasvavien mikrobien ominaisuuksia siten, että amebat voivat lisätä niiden kasvua ja elinkykyä, sekä vaikuttaa näiden tulehdusvasteita aiheuttaviin ominaisuuksiin. On siis mahdollista, että amebat voivat olla epäsuorasti osallisia kosteusvauriorakennuksiin yhdistetyissä terveyshaitoissa. Tarvitaan kuitenkin lisää sekä kokeellista että epidemiologista tutkimusta, jotta amebojen osuus kosteusvaurioituneissa rakennuksissa tapahtuvassa altistumisessa selviäisi.

Avainsanat: Amebat, *Acanthamoeba polyphaga*, bakteerit, sienet, kosteusvaurio, rakennus, yhteiskasvatus, sytokiinit, NO

CONTENTS

ABBREVIATIONS

ANOVA	Analysis of variance
DG-18	Dichloran Glycerol agar, a growth medium for fungi with lower moisture requirements
ELISA	Enzyme-linked immunosorbent assay
Free-living amoebae (FLA)	Environmental amoebae that survive and grow without a host organism
Hagem	Rose Bengal malt extract agar, a colony size restrictive growth medium for hydrophilic fungi
HBSS	Hank's Balanced Salt Solution, a cell substrate solution
IL-6	Interleukin 6 cytokine, an inflammatory marker
2 % MEA	2 % Malt Extract agar, a growth medium for hydrophilic fungi
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, reagent used in cytotoxicity test
NNA	Non-nutritive agar, an amoebal growth medium
NNA-method	A method for detecting amoebae with non-nutritive agar plates and roughly estimating their abundance
NO	Nitric oxide, an inflammatory marker
PYG	Peptone Yeast Glucose broth, an amoebal growth medium
RAW264.7	A mouse macrophage cell line
TNFα	Tumor Necrosis Factor alpha cytokine, an inflammatory marker
TYG	Tryptone Yeast Glucose agar, a bacterial growth medium

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Yli-Pirilä T., Kusnetsov J., Haatainen S., Hänninen M., Jalava P., Reiman M., Seuri M., Hirvonen M.-R., Nevalainen, A. 2004. Amoebae and other protozoa in material samples from moisture-damaged buildings. Environmental Research 96:250-256.

II Yli-Pirilä T., Kusnetsov J., Hirvonen M.-R., Seuri M., Nevalainen A. Survival of amoebae on building materials. 2009. Indoor Air 19:113-121.

III Yli-Pirilä T., Kusnetsov J., Hirvonen M.-R., Seuri M., Nevalainen A. 2006. Effects of amoebae on the growth of microbes isolated from moisture-damaged buildings. Canadian Journal of Microbiology 52:383-390.

IV Yli-Pirilä T., Huttunen K., Nevalainen A., Seuri M., Hirvonen M.-R. 2007. Effects of co-culture of amoebae with indoor microbes on their cytotoxic and proinflammatory potential. Environmental Toxicology 22:357-367.

1 INTRODUCTION

More than 20 years of research has demonstrated that excess moisture and concurrent microbial growth in buildings is associated with adverse health effects suffered by the occupants (IOM 2004). Excess moisture can enter building structures in many ways, for example from leaks in the roof or plumbing, by capillary rise of ground moisture, or by condensation due to inadequate ventilation or thermal or water proofing. The excess moisture can cause damage and facilitate microbial growth in building structures, structural components or on the surfaces of the materials (Haverinen 2002).

Different aspects of the exposure linked with moisture damage have been studied, such as microbial diversity and the presence of volatile organic compounds emitted by these microbes and moistened building materials, but no causative relationships between the experienced health problems and the exposure have been conclusively revealed so far (Bornehag et al. 2001). Studies on the microbes have concentrated on the fungi, and to some extent also on the bacteria present in moisture-damaged environments. Other organisms possibly present and possibly indicating moisture-damages have rarely been studied. However, it is likely that higher organisms able to consume fungi and bacteria as nutrition are also a part of the microbial network present at the moisture and mold damaged building materials. These higher organisms in this respect could include protozoa, such as amoebae, flagellates, ciliates, and even arachnids and insects (Flannigan 2001).

Species of amoebae, flagellates and ciliates are ubiquitous in natural environments containing water. A gram of soil typically contains 10^4 to 10^5 cells of amoebae and flagellates (Ekelund and Rønn 1994) and a liter of natural water may be home to 10^5 to 10^6 protozoal cells (Zimmermann 1997). Because of their ubiquity, amoebae and other protozoa may also be transported into various man-made environments such as buildings. Possible routes of entry may include the remains of water or soil in soles of shoes, or via airborne route through doors, windows and other ventilation shafts. The availability of moisture determines whether amoebae can take up residence in a particular site inside a building. In contrast, the lack of food rarely prevents their growth, as these organisms can utilize a large variety of nutritional sources ranging from bacteria to algae. Amoebae require a water film to become active, that is to feed, move and replicate. The thickness of this water film depends on the size of the organism and can be as low as 5 μm. Therefore, some amoebae and other protozoa should be able to grow on moistened building materials. Many amoebae can survive even if the material dries out because many of the species are able to metamorphose

into resistant forms, cysts, in unfavorable environmental conditions like drought (Hausmann et al. 2003).

The need to determine how common amoebae really are in moisture-damaged buildings arose from a case of a moisture-damaged hospital where several employees suffered serious adverse health symptoms. In the search of the cause for these severe symptoms, a thorough clinical testing was performed on the employees including the presence of IgG and IgA antibodies to *Chlamydophila pneumoniae* (Seuri et al. 2005). Surprisingly, 16 of total of 18 employees were positive for *C. pneumoniae* even though no clinical chlamydial infections had been observed. This phenomenon was thought to be possibly linked with the exposure to the conditions in the moisture-damaged building. However, *C. pneumoniae* and other *Chlamydia*-related bacteria require a host organism and should not be able to survive as such on the moistened building materials. On the other hand, amoebae are natural hosts and carriers of *Chlamydia*-related bacteria (Amann et al. 1997; Birtles et al. 1997; Fritsche et al. 2000). It was considered possible that there were amoebae present in the moisture-damaged sites, and that the elevated antibody levels could be caused by *Chlamydia*-related bacteria residing inside of the amoebae (Seuri et al. 2005). Furthermore, over the years, amoebae had been occasionally detected in samples from buildings with suspected moisture-damage in routine cultivation for fungi and bacteria (unpublished observation). This led to a series of investigations focussed on amoebae in moisture-damaged buildings and building materials, the results of which are presented in this thesis.

In order to elucidate the potential significance of amoebae in the exposure associated with moisture-damaged buildings, one necessary first step was to clarify the role of amoebae as members of the microbial network in the moisture-damaged environment. In this study, the occurrence of amoebae in moisture-damaged buildings is investigated, the ability of amoebae to grow on a selection of building materials is tested, and the effects of amoebae on the growth, viability, cytotoxicity, and proinflammatory potential of indoor bacteria and fungi are assessed.

2 REVIEW OF THE LITERATURE

2.1 Moisture-damage in buildings

2.1.1 The exposing agents and health effects associated with moisture-damaged buildings

The exposure in moisture-damaged buildings is a complex phenomenon in which both biological and chemical exposing agents may be released into the indoor air either in gaseous form or as attached to particles. The agents include spores and cells of microfungi, bacteria, yeasts, mites, protozoa, and their fragments; also toxins and other products of these organisms' metabolism can be present in this multi-faceted exposure (Andersson et al. 1997; Glushakova et al. 2004; Hyvärinen et al. 2002; Nevalainen et al. 1991; Pasanen et al. 1992; Piecková and Wilkins 2004; Van Strien et al. 1994). Furthermore, moisture may also cause chemical reactions in the building materials resulting in release of volatile organic compounds (Korpi et al. 1998). Thus, quantifying the exposure is difficult, and the methods used today - such as measuring the concentration of viable microfungi in indoor air - can only be considered as surrogates of the actual exposure (Nevalainen and Seuri 2005). Nevertheless, the dampness-related exposure has been clearly shown to be associated with adverse health effects for those exposed (Bornehag et al. 2001; IOM 2004). It is also evident that the experienced health effects clearly differ from each other in different buildings with moisture damage, suggesting that the causes for the symptoms are probably not identical (Nevalainen and Seuri 2005). Due to the complexity of the exposure, it is still not known which individual agents cause certain symptoms and what are the pathophysiological mechanisms of the resulting reactions.

The adverse health effects reported in association with mold and moisture damage in buildings are diverse ranging from irritation of eyes to tiredness and general malaise. The most often reported health outcomes are irritation symptoms, repeated respiratory infections and unspecific general symptoms (IOM 2004). The most convincing evidence on the association between the symptoms and exposure to mold and damp has been evaluated for cough, wheeze, dyspnoea and worsening of symptoms of asthma (IOM 2004; Peat et al. 1998). The risk of developing new asthma has been shown to be related to being exposed to moisture-damaged buildings (Jaakkola et al. 2002; 2005; Pekkanen et al. 2007). Table 2.1 describes

examples of mold- and moisture-associated health effects experienced by both adults and children.

Table 2.1 Common symptoms reported in moisture-damaged buildings

	Health effects (examples of references)
Symptoms at the interface of human and the environment	**eyes:** irritation (Meyer et al. 2004; Pirhonen et al. 1996) **skin:** rash, itch, eczema (Engvall et al. 2002; Kilpeläinen et al. 2001; Koskinen et al. 1999b; Park et al. 2006; Simoni et al. 2005) **upper airways:** hoarseness, blocked nose, nasal hyperreactivity (Engvall et al. 2002; Kilpeläinen et al. 2001; Koskinen et al. 1999a, b; Park et al. 2006; Pirhonen et al. 1996; Simoni et al. 2005; Tham et al. 2007) **lower airways:** cough, wheezing, shortness of breath (Belanger et al. 2003; Cho et al. 2006; Dales et al. 1991; Engvall et al. 2002; Gent et al. 2002; Koskinen et al. 1999a, b; Park et al. 2006; Salo et al. 2004; Simoni et al. 2005) **respiratory infections:** common cold, bronchitis (Bakke et al. 2007; Kilpeläinen et al. 2001; Koskinen et al. 1999a, b)
General symptoms	**fever** (Pirhonen et al. 1996) **neuropsychiatric symptoms:** tiredness, lack of concentration, depression (Crago et al. 2003; Ebbehøj et al. 2005; Engvall et al. 2002; Gordon et al. 2004; Kilburn 2003; Koskinen et al. 1999a, b; Pirhonen et al. 1996; Shenassa et al. 2007) **pain:** headache, backache, stomach ache (Ebbehøj et al. 2005; Meyer et al. 2004; Pirhonen et al. 1996) **nausea** (Koskinen et al. 1999a)
Asthma	development of asthma or asthmatic symptoms (Pekkanen et al. 2007) risk of asthma (Bornehag et al. 2005; Jaakkola et al. 2002; 2005; Kilpeläinen et al. 2001; Matheson et al. 2005; Peat et al. 1998; Simoni et al. 2005) worsening of the symptoms of current asthma (Burr et al. 2007; Dharmage et al. 2002; Ly et al. 2008)
Other	aching joints, rheumatoid symptoms (Luosujärvi et al. 2003; Myllykangas-Luosujärvi et al. 2002) hypersensitivity pneumonitis (Temprano et al. 2007)

The mechanisms behind the adverse health effects associated with moisture-damaged buildings are inadequately understood. Many reported symptoms mimic

allergic reactions and IgE-mediated allergy can play a role in the development of symptoms (Horner et al. 1995). However, allergy to fungi, as such, is rare in association with the exposure to damp buildings, with only 5 % of estimated prevalence among the exposed (Immonen et al. 2001; Taskinen et al. 1997). Many studies show that microbes from moisture-damaged buildings can induce inflammatory responses in animal models and *in vitro* (e.g. Hirvonen et al. 1997a; Huttunen et al. 2003; Jussila et al. 2001). Increased levels of inflammatory mediators have also been found in nasal lavage and induced sputum in humans (Hirvonen et al. 1999; Roponen et al. 2001b), suggesting that non-specific inflammation could be an important pathway contributing to the health effects. Other possible mechanisms may be initiated by microbial toxins – many indoor microbes are known toxin-producers and toxins can even be detected in the indoor air of moisture-damaged buildings (Brasel et al. 2005; Gottschalk et al. 2008; Pohland 1993). Immunosuppression due to ciliated cell death and the subsequent impaired particle clearance resulting in higher susceptibility to infections in the airways could be associated with the acute cytotoxicity of indoor microbes shown *in vitro* (Huttunen et al. 2004; Penttinen et al. 2005a; Piecková and Jesenska 1996, 1998). In addition, symptoms like tiredness and depression may be secondary to toxic effects on the central nervous system. Neurotoxicity of the pure mycotoxins has been shown *in vitro* and *in vivo* (Belmadani et al. 1999; Rotter et al. 1996; Stockmann-Juvala et al. 2006). However, it is likely that several different mechanisms may be involved, even simultaneously, since the exposure is complex and the range of experienced symptoms is wide.

2.1.2 Microbial growth in moisture-damaged buildings

Sources and concentrations of indoor microbes

Fungi and bacteria are ubiquitous; they can start growing whenever the environmental conditions allow. Outdoor air, vegetation and soil are the main sources for indoor microbes, although snow cover reduces the outdoor contribution during wintertime in cold climates (Flannigan 2001). Usually, the size of fungal and bacterial propagules is well below 10 µm, which ensures at least their temporary suspension in air currents and subsequent transport into buildings through unfiltered intake air, open windows, doors, and leaks in the building envelope (Górny et al. 1999; Reponen et al. 1994). In addition to outdoor sources, also indoor sources for microbes contribute to the indoor microbial concentrations. Normal daily activities, such as handling of foodstuffs and firewood, release microbes into the indoor air (Lehtonen et al. 1993). Humans themselves are quite a major source of bacteria

(Nevalainen 1989) as also shown by extensive sequencing of house dust bacteria (Rintala et al. 2008).

The indoor air fungal concentrations vary greatly both spatially and temporally, but are mainly at the level of 10^1-10^3 cfu/m^3 in healthy buildings, measured as culturable fungi (Hyvärinen 2002; Meklin 2002; Salonen et al. 2007; Tsai et al. 2007). The bacterial concentrations are slightly higher than the respective fungal concentrations in the indoor air (Hyvärinen 2002; Salonen et al. 2007; Tsai and Macher 2005). The airborne fungal concentrations in moisture-damaged buildings are somewhat higher than those in healthy buildings, on average at the level 10^2-10^3 cfu/m^3 in subarctic climate in Finland (Hyvärinen 2002). In the Finnish guidelines for indoor air quality, wintertime fungal concentrations of 100-500 cfu/m^3 are considered indicative of an indoor source, if the fungal genera indicative of moisture damage are simultaneously present (STM 2003). According to the guidelines, concentrations higher than 500 cfu/m^3 in residences are regarded as "high" and possibly require further investigations of the source and possible remediation of moisture damage.

The adverse health effects are associated with dampness, moisture and microbial growth within the building (Nevalainen and Seuri 2005). However, it appears that the increased microbial concentrations in indoor air are not in a causal relationship with the increase of reported adverse health effects (Bornehag et al. 2004; Nevalainen and Seuri 2005). In many occupational environments, the microbial concentrations are several orders of magnitude higher than those found in homes or offices (e.g. Mackiewicz 1998). Instead, the health effects seem to be linked with the conditions that allow the growth of microbes (IOM 2004).

Growth of microbes on moist building materials

The factors affecting microbial growth in buildings are the availability of water, the availability of nutrients, and temperature. Of these three parameters, the availability of water is the critical factor, a general prerequisite for microbial growth. The other factors are usually available. Temperatures in the buildings and within the building envelope, typically 0-25 ºC, are well in the range of growth for mesophilic fungi (Flannigan and Miller 2001). Fungi and bacteria excel at being able to extract the essential nutrients from seemingly poor environments. Water, dust and other (organic) materials accumulating on the building material surfaces provide enough substrates to microbial growth, and some building materials themselves may include nutrients suitable for micro-organisms (Flannigan and Miller 2001).

The amount of available water, often described as water activity (a_w), can discriminate which microbial species will thrive on the moistened building material.

Certain fungi, such as *Aspergillus versicolor*, can survive and grow over a wide range of a_w, whereas others such as members of the genus *Trichoderma* require high a_w to grow and sporulate. Thus, the microbial flora detected may give a clue on whether the material in question is only partly moistened or whether it is thoroughly wet. The microbes which are indicative to moisture/dampness and do not belong to normal flora of indoor air include the fungi *A. versicolor*, *A. fumigatus*, *Trichoderma*, *Exophiala*, *Phialophora*, *Ulocladium*, *Fusarium*, *Wallemia*, *Stachybotrys*, yeasts, and the gram-positive bacteria, actinomycetes (Samson et al. 1994). In addition to fungi and bacteria, there are many other organisms growing on damp building materials, such as protozoa, but these have been given little attention so far.

2.1.3 Proinflammatory and cytotoxic responses induced in vitro by microbes from moisture-damaged buildings

The proinflammatory and cytotoxic potential of a microbe is related to its ability to evoke inflammation and tissue damage. This potential can be studied *in vitro* by exposing cell lines, such as macrophages and epithelial cells originating from humans, mice or rats, to known doses of the microbe or its metabolites. Such an exposure can induce defence functions in the cells, for example the release of inflammatory markers or even cause cell death, which in turn can be measured to estimate the effect of the exposing agent. Macrophages and epithelial cell lines are the primary cell types against inhaled particles in the lung, and therefore often used in studies investigating the effects of indoor microbial contaminants (Hirvonen et al. 1997a).

Inflammatory markers include nitric oxide (NO) and a complex network of cytokines such as interleukins and tumor necrosis factors. Cytokines are soluble proteins of low molecular weight, whereas NO is a gaseous radical. They are important mediators in the host defense system against inflammatory stimuli and each plays a specific role in this process. The inflammatory markers examined in this thesis include NO, interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα). These markers were selected because previous research on effects of microbes from moisture-damaged buildings revealed these to be the most relevant in describing differences between the potential of indoor microbes (e.g. Hirvonen et al. 1997a, b; 1999).

NO mediates many biological processes, such as airway and vascular tone, and inflammatory cell activation (Fischer et al. 2002; Nevin and Broadley 2002). It is

enzymatically synthesized from L-arginine by NO synthase by a variety of cells including alveolar macrophages and airway epithelial cells (Moncada et al. 1991). NO is rapidly transformed to nitrite and nitrate, and these compounds can be analysed and utilized as a marker of NO production. With respect to the cytokines, TNFα is an early phase macrophage-produced proinflammatory cytokine produced as a part of the non-specific immune response. TNFα can enhance the production of other cytokines and NO, and increase the phagocytic activity of cells. TNFα enhances the transfer of neutrophils and monocytes to sites of inflammation by increasing vascular permeability (Sedgwick et al. 2002). TNFα is also involved in cell death by both apoptosis (programmed cell death) and necrosis (uncontrolled cell death) (Barnes et al. 1998; Luster et al. 1999). IL-6, also a proinflammatory cytokine, has a role in both innate and adaptive immunity. It affects the functions of lymphocytes and neutrophils, and stimulates the growth and differentiation of B-cells (Cenci et al. 2001). IL-6 is produced by many different cells, such as macrophages, epithelial cells, and T-cells (Abbas et al. 2000).

Cytotoxicity can be defined as a description of the extent of the destructive or killing capacity of an agent on living cells. Many of the fungi and bacteria isolated from moisture-damaged buildings are cytotoxic *in vitro* (Huttunen et al. 2000; 2001; 2003), and can cause tissue damage in mice lungs (Jussila et al. 2001; 2002a; 2002b; Nikulin et al. 1996; 1997). In humans, inflammation induced lung epithelial cell damage is associated with asthma pathogenesis (Holgate et al. 2003).

In addition to being cytotoxic, indoor fungi and bacteria can induce the production of proinflammatory mediators such as IL-1β, IL-6 and TNF-α *in vitro* (Hirvonen et al. 1997a, b; Huttunen et al. 2000; 2001; 2003). Animal studies conducted on these microbes have also revealed immunostimulation in the lungs of the test animals (Jussila et al. 2001; 2002a; 2002b; 2003). Furthermore, similar biological activity has also been shown for human individuals in nasal lavage or in induced sputum sampling (Hirvonen et al. 1999; Purokivi et al. 2001; 2002; Roponen et al. 2003; Stark et al. 2006).

Many factors can alter the cytotoxicity or proinflammatory potential of the microbes from moisture-damaged buildings. For example, the building material on which the microbe has grown can alter its potency, probably due to the different nutrient and pH conditions present in each building material (Roponen et al. 2001a). Many indoor microbes are able to produce toxins, and the toxin production is possibly influenced by the conditions in which the microbe lives. Microbial toxins have indeed been found on mold-infested building materials (Charpin-Kadouch et al. 2006; Nielsen et al. 1999). Even differences between different brands of the same building material can affect the toxicity of the microbes growing on the material, as shown for the bacterium *Streptomyces californicus* and the fungus *Stachybotrys*

chartarum which were grown on plasterboards of different compositions (Murtoniemi 2003). Furthermore, microbial interaction can alter the biological potency of the counterparts. For example, when *S. californicus* and *S. chartarum* were grown together at the same location, i.e. were co-cultivated, and thus had to compete for the same living space and resources, they produced more potent metabolites than when growing alone. In turn, the cytotoxic and proinflammatory responses that these microbes raised in cells were synergistically higher in samples with co-cultivated *S. californicus* and *S. chartarum* than separately grown microbes (Murtoniemi et al. 2005; Penttinen et al. 2005a; 2006; 2007). Also simultaneous exposure to *S. californicus* and *S. chartarum* can synergistically increase the cytotoxic and proinflammatory responses in cells (Huttunen et al. 2004) and the effect can be dependent on the proportions of these microbes (Penttinen et al. 2005b). Examples of *in vitro* studies on proinflammatory potential of microbes from moisture-damaged buildings are listed in Table 2.2.

Table 2.2. Examples of proinflammatory and cytotoxic effects of microbes from moisture-damaged buildings *in vitro*

Exposing microbes	Cell type	Effects (reference)
"high" microbial exposure (24-h personal filter sampling)	RAW264.7[1]	Increased production of IL-1β, IL-6 and TNF-α compared to "low" exposure (Roponen et al. 2003)
Streptomyces sp.	RAW264.7	Increased production of TNF-α, IL-6, with subsequent NO production (Hirvonen et al. 1997a, b)
Streptomyces anulatus	A549[2]	Production of NO and IL-6 (Jussila et al. 1999)
Stachybotrys chartarum *Aspergillus versicolor* *Penicillium spinulosum*[4]	RAW264.7 A549 28SC[3]	Cytotoxic to cells, only minor production of cytokines and NO (Huttunen et al. 2003)
Pseudomonas fluorescens *Streptomyces californicus* *Bacillus cereus*[4]	RAW264.7 A549 28SC	Cytotoxic to cells, production of NO and cytokines TNF-α, IL-6 and IL-1β (Huttunen et al. 2003)
Mycobacterium avium (two strains) *Mycobacterium terrae* (two strains)	RAW264.7 A549 28SC	Production of TNF-α and IL-1β (only in RAW264.7), IL-6 and NO (all cell lines); mildly cytotoxic to RAW264.7, not toxic to human cell lines (Huttunen et al. 2001)
Aspergillus fumigatus	RAW264.7	Increase in mRNA expression of TNF-α, MIP-1α, MIP-1β, and MCP-1 (Pylkkänen et al. 2004)
simultaneous exposure to *Streptomyces californicus* and *Stachybotrys chartarum*	RAW264.7	Synergistic increase in production of IL-6 (Huttunen et al. 2004), MIP2, and TNF-α, and cytotoxicity (Penttinen et al. 2005b)
co-cultivated *Streptomyces californicus* and *Stachybotrys chartarum*	RAW264.7	Synergistically increased apoptosis and cell cycle arrest (Penttinen et al. 2005a) Stimulation of production of cytostatic compounds (Penttinen et al. 2006) Production of genotoxic metabolite causing DNA damage and genotoxic responses (Penttinen et al. 2007)

[1]Mouse macrophages, [2]Human alveolar epithelial cells, [3]Human macrophages, [4]Microbes listed in rank order of potency from highest to lowest

2.2 Amoebae

Amoebae are single-celled eukaryotic protozoans that belong to groups that can be genetically rather distant from each other. Amoebae do not constitute a single taxonomic category; the term “amoeba” is rather a practical term that refers to cells that are able to move and engulf food particles by producing projections of the cytoplasm. This amoeboid behavior is common among the eukaryotes. However, amoeboid cells represent morphologically very diverse forms of living organisms. For example, there are amoebae with one or more flagella (e.g. *Naegleria*), amoebae with different types of shells (e.g. *Euglypha*), amoebae that are deeply branched (e.g. *Stereomyxa*) and those that are more or less conical (e.g. *Mayorella*) (Hausmann et al. 2003). To add to the diversity, even a single cell can have several morphotypes depending on its environment. Even more variation can be found in the survival strategies; for example, there are amoeboid cells that are strictly parasitic (e.g. *Entamoeba*) (Stauffer and Ravdin 2003), those that can photosynthesize (e.g. *Chlorarachnion*) (McFadden et al. 1994), and opportunistic organisms that can alternatively hunt for their food or become parasites of animal hosts (e.g. *Acanthamoeba*) (De Jonckheere 1991).

The taxonomy of amoebae, as well as the taxonomy of all protists, is undergoing a process of rediscovery. The traditional ultrastructural methods are being complemented with biochemical identification and DNA-based methods and this has brought new insights into the relationships between the protists. Although the amoebal species dictated close morphologically have often been found to be so also phylogenetically, the relationships between the higher taxonomical orders are not quite so stable (Hausmann et al. 2003). Currently, the newest taxonomical system proposed by the International Society of Protozoologists (Adl et al. 2005) divides all eukaryotes into six clusters. Amoeboid organisms are present in almost all of these groups. The group of amoebae most interesting within the scope of this study are, however, the free-living amoebae.

2.2.1 Free-living amoebae

Free-living amoebae are either heterotrophic or opportunistically parasitic amoebae which are ubiquitous in a wide range of natural and man-made microhabitats all over the world (Rodríguez-Zaragoza 1994). Most of the species are able to exist in durable resting forms, cysts, in which the organism endures adverse conditions.

Even though most genera of the free-living amoebae can be found practically everywhere, some are principally marine organisms, some are most common in fresh water, and some are predominant in soil. Examples of free-living amoebae and the environments in which they are encountered are listed in Table 2.3.

Free-living amoebae and other protozoa have a significant role in the ecosystem in the mineralization of nitrogen, carbon and phosphorus due to their importance as bacterial predators (Ekelund and Rønn 1994; Rodríguez-Zaragoza 1994; VreekenBuijs et al. 1997). It should be noted that although amoebae often feed on bacteria, bacteria can also utilize amoebae as a vehicle for survival, replication, or even as means of transmission from one host to another. For example, legionellae are able to avoid digestion in amoebae, in fact they can replicate intracellularly in amoebae until the amoebae burst (Newsome et al. 1998). Even though bacteria are often the most palatable nutrition for many free-living amoebae, these organisms are versatile in their feeding habits and they can feed on ciliates, other amoebae, fungal spores and even hyphae, and many species are also able to grow in axenic nutrient broths (Gilbert et al. 2003; Hausmann et al. 2003; Schuster 2002).

The most well-known and most studied free-living amoebae are the genera *Acanthamoeba* and *Naegleria*; this is perhaps because species of these genera have been associated with disease in humans, either directly or as carriers of pathogenic bacteria. Acanthamoebae are small soil amoebae, 25 to 40 µm in diameter that are able to form strong double-walled cysts within a time course of 40 hours (Aksozek et al. 2002; Chagla and Griffiths 1974; Chávez-Munguía et al. 2005; Sykes and Band 1985; Turner et al. 2004). Numerous species of acanthamoebae have been described, such as *A. polyphaga*, *A. castellanii*, and *A. culbertsonii* (Page 1988), but this differentiation has been performed on a morphological basis. Newer genetic methods do not unambiguously support this division and since the 1990s many papers refer to acanthamoebae rather as sequence types T1 to T15 according to their nuclear small ribosomal subunit RNA genes (SSU rDNA) (De Jonckheere 2007; Gast et al. 1996; Hewett et al. 2003; Stothard et al. 1998). This division has been supported by immunological patterning (Walochnik et al. 2001) and the current opinion on the subgenus systematics for acanthamoebae states that species names should be replaced with genotype numbers until the phylogenetical status of each species can be resolved.

Table 2.3 Examples of free-living amoebae and their habitats

Genus	Isolated from (selected references)	Main habitat	Pathogenicity
Acanthamoeba	**Humans:** Human eye (Visvesvara et al. 1975); human brain (Martínez et al. 1977); human nasal passages/mucosa (De Jonckheere and Michel 1988; Sadaka et al. 1994) **Human environments:** Drinking water (Hoffmann and Michel 2001; Michel et al. 1995a; Shoff et al. 2008); house dust in bathroom (Seal et al. 1992); swimming pools, whirlpools, physiotherapy tubs (De Jonckheere 1979b; Rivera et al. 1993; Vesaluoma et al. 1995); sanitary areas in hospital (Rohr et al. 1998); terrariums and aquariums (De Jonckheere 1979a; Hassl and Benyr 2003); contact lens cases (Larkin et al. 1990); eyewash stations (Tyndall et al. 1987); dental units (Barbeau and Buhler 2001); sewage sludge (Griffin 1983) **Animals:** Intestines of bull, rabbit, pigeon, and turkey (Kadlec 1978); reptile intestines (Hassl and Benyr 2003); fish (Dyková and Lom 2004); toucan (Visvesvara et al. 2007) **Soils:** Arable soil (Sawyer 1989); desert topsoil crust (Bamforth 2004); forest soil and litter (Rodríguez-Zaragoza et al. 2005) **Water:** Ocean, brackish and fresh water sediments (Sawyer et al. 1977); marine water (Arias Fernandez et al. 1989); fresh water (Befinger et al. 1986; Ettinger et al. 2003; Mansour et al. 1991); natural hot springs and thermal waters (Lekkla et al. 2005; Rivera et al. 1989) **Other:** Surface of edible mushrooms (Napolitano 1982), fresh vegetables (Rude et al. 1984), air samples (Rivera et al. 1987)	Soil	Established opportunistic pathogen
Balamuthia	**Humans:** Human brain (Visvesvara et al. 1993) **Human environments:** Soil in indoor potted plant (Schuster et al. 2003), soil in outdoor potted plant (Dunnebacke et al. 2004) **Animals:** Brain of gorillas and other primates (Rideout et al. 1997; Visvesvara et al. 1993) **Soils:** Soil sample (Dunnebacke et al. 2003)	Soil / animals	Established opportunistic pathogen

Hartmanella	**Humans:** Human eye (Aimard et al. 1998; Aitken et al. 1996) **Human environments:** Drinking water (Shoff et al. 2008); dental units (Barbeau and Buhler 2001), swimming pools and physiotherapy tubs (Rivera et al. 1993; Vesaluoma et al. 1995), hot water system and sanitary areas in hospital (Rohr et al. 1998) **Animals:** Dog, turkey (Kadlec 1978); fish (Dyková and Lom 2004) **Soils:** Desert topsoil crust (Bamforth 2004); **Water:** Marine sediments (Anderson et al. 1997) **Other:** Laboratory cell cultures (Fogh et al. 1971), air samples (Lawande 1983)	Soil	Possibly a pathogen
Naegleria	**Humans:** Human brain (Carter 1970), nasal passage of healthy humans (Sadaka et al. 1994) **Human environments:** Drinking water (Hoffmann and Michel 2001; Michel et al. 1995a), aquariums (De Jonckheere 1979a), swimming pools (De Jonckheere 1979b; Rivera et al. 1993), dental units (Barbeau and Buhler 2001), sanitary areas in hospital (Rohr et al. 1998) **Animals:** Fish (Dyková and Lom 2004), tapir (Lozano-Alarcón et al. 1997), **Water:** Fresh water (Mansour et al. 1991), **Other:** Laboratory cell cultures (Fogh et al. 1971), air samples (Lawande 1983; Rivera et al. 1987)	Water	Established opportunistic pathogen
Vannella	**Human environments:** Drinking water (Shoff et al. 2008); dental units (Barbeau and Buhler 2001), sanitary areas in hospital (Rohr et al. 1998), **Animals:** Fish (Dyková and Lom 2004); **Soils:** Desert topsoil crust (Bamforth 2004) **Water: S**urface of algae in coastal marine water (Armstrong et al. 2000), brackish water pond (Anderson 1998) **Other:** Air sample (Rivera et al. 1987)	Fresh water / soil / marine water	Not shown

***Vahlkampfia* / vahlkampfids**	**Humans:** Human eye (Aitken et al. 1996; Alexandrakis et al. 1998) **Human environments:** Drinking water (Shoff et al. 2008); dental units (Barbeau and Buhler 2001), swimming pools and physiotherapy tubs (Rivera et al. 1993), contact lenses (De Jonckheere and Brown 2005), hot water system and sanitary areas in hospital (Rohr et al. 1998) **Animals:** Pig, turkey (Kadlec 1978) **Soils:** Desert topsoil crust (Bamforth 2004), agricultural soil (Brown and De Jonckheere 2004) **Water: F**resh water (Mansour et al. 1991), cold fresh water (Robinson et al. 2007), marine water (Munson 1992), marine sediment (Anderson et al. 1997) **Other:** Air sample (Rivera et al. 1987)	Fresh water / marine water	Not shown
Amoeba	**Human environments:** Cool-mist humidifier (van Assendelft et al. 1979), swimming pool (Rivera et al. 1983) **Soils:** Desert topsoil crust (Bamforth 2004), gut of earthworms (Parthasarathi et al. 2007)	Fresh water	Not shown
Mayorella	**Human environments:** Drinking water (Shoff et al. 2008) **Animals:** Gills of fish (Bermingham and Mulcahy 2007) **Soils:** Desert topsoil crust (Bamforth 2004) **Water:** Antarctic ocean (Mayes et al. 1997), marine water (Anderson 1998)	Fresh / marine water	Not shown
Saccamoeba	**Human environments**: Drinking water (Shoff et al. 2008); hot water system in hospital (Rohr et al. 1998), aquariums (Mrva 2007) **Soils:** Desert topsoil crust (Bamforth 2004) **Water:** Fresh water (Mrva 2007), marine sediments (Anderson et al. 1997) **Other:** Air samples (Rivera et al. 1987)	Soil / fresh water	Not shown

Acanthamoebae are one of the most ubiquitous amoebae dispersed throughout the whole world. Acanthamoebae are very adaptive organisms and they have a potential to produce an impressive selection of exoenzymes that might help extracting nutrients from a variety of growth environments (Anderson et al. 2005). Acanthamoebae are often carriers of intracellular bacteria; these bacteria may either be harmful for the amoeba or they may enhance amoebal growth (Collingro et al. 2004). Some of the intracellular bacteria, such as *Legionella* or *Chlamydia*, can be pathogenic to humans. Acanthamoebae have been isolated in almost every conceivable environment, ranging from the intestines of reptiles (Hassl and Benyr 2003) to Dry Valleys' soil of Antarctica (Bamforth et al. 2005). Thus, it is very likely that acanthamoebae would also be present in moisture-damaged building materials.

Acanthamoebae feed mainly on bacteria, but given the opportunity, they can also invade and parasitize animal hosts. Acanthamoebae are opportunistic organisms and most often take advantage of hosts with compromised immunocompetence, but a few infections of healthy individuals have also been reported. In humans, two main types of acanthamoeba infections occur. Acanthamoebae can be causative agents of fatal granulomatous amoebic encephalitis in immunocompromised humans (Martínez et al. 1977). However, the incidence of this disease is low, with only 60 cases reported to the date since the sixties (WHO 2003). Another common, but still rare, acanthamoeba-caused infection is the severe keratitis associated with the use, or rather the misuse, of contact lenses (Visvesvara et al. 1975). Poor hygiene practices are the main risk factor for acanthamoeba keratitis, but also swimming while wearing the contact lenses may expose the individual to acanthamoeba infection. In the United States, the incidence of acanthamoeba keratitis is approximately 1-2 cases per million contact lense wearers (CDC 2007; WHO 2003). Thus, infections are extremely rare, even though humans and acanthamoebae cross paths constantly because of the universal nature of the amoeba.

Although these human infections have increased the research on acanthamoeba, the characteristics of the organism explain its common occurrence in the scientific literature. If a model for environmental amoebae is needed in laboratory experiments, acanthamoebae are often selected because they are readily adapted to axenic (without microbes as a substrate) culture media (Jensen et al. 1970; Schuster 2002), and easily maintained and controlled as they have a simple life cycle and form cysts. Acanthamoebae reproduce by binary fission and they have a rather high growth rate even in axenic growth media (Byers et al. 1980). Due to their many applications, there is an abundance of acanthamoebal strains both pathogenic and non-pathogenic available at culture collections.

Another common group of free-living amoebae are *Naegleria*, which are found in fresh water and soil. In addition to the feeding form and the cyst formation, many of the naegleriae can live in a non-feeding locomotive flagellated stage. Over 20 species of naegleriae have been described, of which the thermotolerant species *N. fowleri*, *N. australiensis* and *N. italica* can be pathogenic (De Jonckheere 2002). So far, only one species of the naegleriae, *N. fowleri*, has been shown to cause primary amoebic encephalitis (PAM) in previously healthy humans. After infection, death occurs rapidly, almost invariably within 10 days. The PAM cases are usually associated with swimming in warm water where naegleriae flourish. Although much studied from the point of view of pathogenicity to humans, the environmental behavior of naegleriae is not as well known as that of the acanthamoebae. The focus of naegleriae research is still in evaluating the possible exposure routes to the amoebae or in finding an effective cure for PAM. Recently, also the ecological role of naegleriae has been examined (Declerck et al. 2005; 2007; Xinyao et al. 2006). In a similar manner to acanthamoebae, naegleriae can be associated with intracellular bacteria (Newsome et al. 1985; Walochnik et al. 2005).

Another well-known amoebal genus is *Dictyostelium*, especially *D. discoideum*, a soil amoeba also known as "cellular slime mold", that forms multicellular structures of tens of thousands of cells in adverse conditions (Hausmann et al. 2003). This genetically malleable amoeba has a unique life cycle that employs several cellular processes and biochemical mechanisms such as cytokinesis, chemotaxis, signal transduction and cell sorting. These make the amoeba a popular model organism for biomedical and molecular biology research, since these phenomena are absent or less readily accessible in other biological models (Chisholm et al. 2006). Other free-living amoebae that are occasionally found in human environments are *Hartmanella*, *Vanella*, *Saccamoeba*, and the first amoeba to ever be described scientifically, *Amoeba*. These amoebae have been sporadically studied in the laboratory conditions but more often they have been examined in studies of the biodiversity of the protozoa in different environments.

Limits of the survival of amoebae

Like most micro-organisms, amoebae are ubiquitous in various environments throughout the Earth. Some amoebae seem to survive for extended periods of time in their cyst form under very harsh environmental conditions, only growing whenever the conditions allow. The cyst-forming amoebae seem to be tolerant of a wide range of temperatures: viable cysts and even trophozoite forms of amoebae have been isolated at cold temperatures in the Antarctic (Bamforth et al. 2005; Brown et al. 1982), but also can withstand the heat of natural hot springs (Baumgartner et al.

2003; Lekkla et al. 2005). In laboratory conditions, hartmanellae and saccamoebae have been cultured even at 53 ºC (Rohr et al. 1998). There is only little information of the pH tolerance of amoebae. Based on the scarce information available, it seems that these organisms are well adapted to a range of pH values. Viable amoebae have been isolated from waters with pH fluctuating between 3.6 and 8.4 (Sykora et al. 1983), and from lime-alkali waters in two lakes in Kenya (Bamforth et al. 1987).

In this context, the environmental conditions on moisture-damaged building materials do not restrict the survival for amoebae in general. The temperature is usually well above 0 ºC and below 40 ºC throughout the building envelope with the possible exception of the outer wall. The alkalinity/acidity of water films and moisture on building materials has rarely been studied but from what studies are available, it seems that the pH of most building material extracts varies from being slightly acidic to neutral. For example, the pH of pine plywood extract is approximately 6 (Lebow and Winandy 1999) and that of gypsum board near to 7 as reported by safety data sheets of gypsum board products of National Gypsum company in 2007 (www.nationalgypsum.com). The pH values of extracts of oven-heated pulverized flour of different species of wood varied between 4.13 (red pine) and 5.15 (aspen) (He and Yan 2005), but it is questionable how well these values relate to the pH values present in the raw material. On the other hand, the pH value of 12 of pore water within concrete (Pavlík 2000) is very alkaline and may not allow amoebal survival. Summing up, it seems that moisture is the critical factor determining whether for amoebae will grow in buildings, just as it is for bacteria and fungi.

2.2.2 Amoebae and bacteria

Amoebae and bacteria exist in a close embrace wherever they meet. There are many possible outcomes of this interaction; for example when amoebae phagocytize bacteria, they can be digested, but instead some bacterial species can avoid digestion and stay viable inside the amoebae. The viable intracellular bacteria may be expunged after some time, or digested later, or become endosymbionts or parasites of the amoeba. Some bacteria, like legionellas and chlamydiales, can even utilize amoebae as their vehicle for replication and transmission (Corsaro and Venditti 2004; Newsome et al. 1998). The bacteria that are not consumed by amoebae are denoted as “amoeba-resisting bacteria” (Greub and Raoult 2004). Examples of the host amoebae and amoeba-resisting bacteria are listed in Table 2.4. The relationship between certain bacteria and amoebae does not necessarily remain stable. There are several examples of interactions that might under some environmental conditions be

lethal to bacteria, and in different conditions lethal to the amoeba. For example, depending on the environmental conditions, a previously harmless endosytobiont may turn parasitic and kill the amoeba (Cirillo et al. 1997; Greub et al. 2003; Lebbadi et al. 1995).

Alternatively, the interactions between the bacteria and the amoebae could also be seen as a continuum of different stages in evolution from antagonism to symbiosis, the ultimate goal of stable relationship beneficial to both (Cirillo 1999; Jeon 1995). For example, Jeon (1995) demonstrated that when an unidentified gram-negative "X-bacterium", later defined as *Legionella jeonii* (Candidatus) (Park et al. 2004), infected a strain of *Amoeba proteus*, the bacterium was originally very cytotoxic to the amoeba. However, some amoebae survived and within a period of 18 months, the bacterium had turned into being an obligate endosymbiont of the amoeba so that it was then necessary for the survival of the amoeba. Several physiological and genetical changes occurred in both of the species during this adaptation (Jeon 2004; Jeon and Jeon 2004). A similar phenomena was induced for *Dictyostelium discoideum* and *Escherichia coli*; in this experiment both of the species lost their pure culture identities within two years (Todoriki and Urabe 2006).

Many amoebae have been found to carry intracellular bacteria and it has been suggested that as many as 20 % of the environmental and clinical amoebal strains may harbor internal bacteria (Fritsche et al. 1993). There are two terms used in the literature describing the intracellular bacteria in the host amoebae: "endosymbiont" and "endosytobiont". The use of these terms overlap, but the term "endosytobiont" is more often used for bacteria that can also be cultured outside of the amoebae, thus being facultative intracellular bacteria. These bacteria can sometimes cause the death of the amoeba. However, other intracellular bacteria are obligate endosymbionts that cannot be cultivated outside of the amoebae (e.g. Amann et al. 1997; Drozanski 1991; Fritsche et al. 1993). It is not always clear whether these endosymbionts are obligate for the survival of amoebae. In many cases, the researchers have not been able to kill the bacteria within the host so that the host survives (Hall and Voelz 1985; Molmeret et al. 2005). Whether the death of the amoeba is due to the loss of essential endosymbionts or due to the harmful effect of the antibiotic, remains a question of debate.

Table 2.4 Bacteria that are able to survive or grow inside of amoebae

Relationship of the bacterium to amoeba	Bacterium	Amoeba	Reference(s)
Obligate intracellular parasites or endosymbionts	Chlamydia-related bacteria	*Acanthamoeba* spp.	(Amann et al. 1997; Birtles et al. 1997; Fritsche et al. 2000)
	Candidatus "*Protochlamydia amoebophila*"	*Acanthamoeba* spp.	(Collingro et al. 2005)
	Candidatus "*Procabacter acanthamoebae*"	*Acanthamoeba* spp.	(Horn et al. 2002)
	Candidatus "*Amoebiphilus asiaticus*"	*Acanthamoeba* spp.	(Horn et al. 2001)
	Candidatus "*Odyssella thessalonicensis*"	*Acanthamoeba* sp.	(Birtles et al. 2000)
	Candidatus "*Paracaedibacter acanthamoebae*"; C. "*P. symbiosus*"	*Acanthamoeba* sp.	(Horn et al. 1999)
	Candidatus "*Caedibacter acanthamoebae*"	*Acanthamoeba* sp.	(Hall and Voelz 1985; Horn et al. 1999)
	Candidatus "*Legionella jeonii*"	*Amoeba proteus*	(Jeon 1995; Park et al. 2004)
	Legionella lyticum (comb. nov.)	*Acanthamoeba castellanii*	(Drozanski 1991; Hookey et al. 1996)
	Legionella drancourtii (sp. nov.)	*Acanthamoeba polyphaga*	(La Scola et al. 2004a)
	Rickettsiales-like	*Acanthamoeba* spp.	(Fritsche et al. 1999)
	Caedibacter-like	*Acanthamoeba* sp.	(Xuan et al. 2007)
	Erlichia-like	*Saccamoeba* sp.	(Michel et al. 1995b)
	Unidentified gram-negative rods	*Acanthamoeba* spp. *Acanthamoeba* sp.	(Fritsche et al. 1993; Yagita et al. 1995)

Table 2.4 continued

Relationship of the bacterium to amoeba	Bacterium	Amoeba	Reference(s)
Natural infection	*Mycobacterium* sp.	*Acanthamoeba* sp.	(Yu et al. 2007)
	Burkholderia pickettii	*Acanthamoeba* sp.	(Michel and Hauröder 1997)
	Cytophaga sp.	*Acanthamoeba* sp.	(Müller et al. 1999)
	Pseudomonas aeruginosa	*Acanthamoeba*	(Michel et al. 1995a)
	Neochlamydia hartmannellae	*Hartmanella vermiformis*	(Horn et al. 2000)
	Legionella drozanskii sp. nov., *L. rowbothamii* sp. nov., *L. fallonii* sp. nov.	*Acanthamoeba polyphaga*	(Adeleke et al. 1996; Adeleke et al. 2001)
	member of the Rickettsia	*Nuclearia pattersoni* sp. n.	(Dyková et al. 2003a)
	Flavobacterium-like bacteria	*Acanthamoeba* sp.	(Horn et al. 2001)
	Legionella-like bacterium	*Unidentified amoeba from soil sample*	(Newsome et al. 1998)
	Two different unidentified species of bacteria in one amoeba	*Naegleria clarki*	(Michel et al. 1999; Walochnik et al. 2005)
	Two bacteria in one amoeba, belonging to groups Parachlamydia and Procabacter	*Acanthamoeba* sp.	(Heinz et al. 2007)

Table 2.4 continued

Relationship of the bacterium to amoeba	Bacterium	Amoeba	Reference(s)
Intracellular replication shown in laboratory	*Simkania negevensis*	*Acanthamoeba polyphaga*, *Acanthamoeba* sp., *Naegleria clarki*, *Balamuthia mandrillaris*, *Hartmanella* spp.	(Kahane et al. 2001; Michel et al. 2005)
	Chlamydia pneumoniae	*Acanthamoeba castellanii*	(Essig et al. 1997)
	Neochlamydia hartmannellae	*Dictyostelium discoideum*	(Horn et al. 2000)
	Waddlia chondrophila	*Hartmanella vermiformis*, *Acanthamoeba* sp., *Vahlkampfia ovis*, *Dictyostelium discoideum*	(Michel et al. 2004)
	Candidatus "*Criblamydia sequanensis*"	*Acanthamoeba castellanii*	(Thomas et al. 2006)
	Francisella tularensis	*Acanthamoeba castellanii*	(Abd et al. 2003)
	Listeria monocytogenes	*Acanthamoeba* sp.	(Ly and Müller 1990)
	Helicobacter pylori	*Acanthamoeba castellanii*	(Winiecka-Krusnell et al. 2002)
	Mobiluncus curtisii	*Acanthamoebae*	(Tomov et al. 1999)
	Burkholderia cepacia complex	*Acanthamoeba polyphaga*	(Landers et al. 2000; Marolda et al. 1999)
	Legionella pneumophila	*Acanthamoeba castellanii*, *Dictyostelium discoideum*, *Hartmanella vermiformis*, *Balamuthia mandrillaris*, *Naegleria lovaniensis*, *Naegleria fowleri* *Willaertia magna*	(Declerck et al. 2005; Dey et al. 2009; Holden et al. 1984; Kuiper et al. 2004; Newsome et al. 1985; Shadrach et al. 2005; Solomon et al. 2000)

Table 2.4 continued

Relationship of the bacterium to amoeba	Bacterium	Amoeba	Reference(s)
Intracellular replication shown in laboratory	*Legionella dumoffii*	*Acanthamoeba castellanii*	(Neumeister et al. 1997)
	Legionella rowbothamii	*Hartmanella vermiformis*	(Adeleke et al. 1996; Adeleke et al. 2001)
	Legionella anisa	*Acanthamoeba polyphaga*	(La Scola et al. 2001)
	Escherichia coli K1	*Acanthamoeba* sp.	(Jung et al. 2007)
	Escherichia coli 0157	*Acanthamoeba polyphaga*	(Barker et al. 1999)
	Afipia felis	*Acanthamoeba polyphaga*	(La Scola and Raoult 1999)
	Salmonella enterica	*Acanthamoeba rhysodes*	(Tezcan-Merdol et al. 2004)
	Salmonella typhimurium	*Acanthamoeba polyphaga*	(Gaze et al. 2003)
	Mycobacterium avium, M. fortuitum, M. marinum, M. kansasii	*Acanthamoeba castellanii*	(Cirillo et al. 1997; Goy et al. 2007; Miltner and Bermudez 2000)
	Mycobacterium marinum, M. avium	*Dictyostelium discoideum*	(Skriwan et al. 2002; Solomon et al. 2003)
	Mycobacterium xenopi	*Acanthamoeba* sp.	(Drancourt et al. 2007)
	Mycobacterium massiliense	*Acanthamoeba polyphaga*	(Adékambi et al. 2004)
	Mycobacterium chelonae, M. fuerthensis, M. monacense, M. neoaurum	*Acanthamoeba polyphaga*	(Pagnier et al. 2008)
	Vibrio cholerae	*Acanthamoeba castellanii*	(Abd et al. 2005; Abd et al. 2007; Saeed et al. 2007; Thom et al. 1992)
	Porphyromonas gingivalis, Prevotella intermedia	*Acanthamoeba castellanii*	(Wagner et al. 2006)

Table 2.4 continued

Relationship of the bacterium to amoeba	Bacterium	Amoeba	Reference(s)
Intracellular replication shown in laboratory	*Pasteurella multocida*	*Acanthamoeba polyphaga*, *Hartmanella vermiformis*	(Hundt and Ruffolo 2005)
	Aquicella lusitana, *A. siphonis*	*Hartmanella vermiformis*	(Santos et al. 2003)
	Alcaligenes sp., *Bradyrhizobium liaoningense*, *B. japonicum*, *Brevundimonas aurantiaca*, *Clostridium frigidicarnis*, *Chromobacterium haemolyticum*, *Chryseobacterium taichungense*, *C. indologenes*, *Delftia tsuruhatensis*, *Flavobacterium sp.*, *Herbaspirillum rubrisubalbicans*, *Methylobacterium fujiwaense*, *Methylobacterium sp.*, *Pantoea ananatis*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. otitidis*, *Rahnella aquatilis*, *Rasbo bacterium*, *Rhodopseudomonas palustris*, *Serratia fonticola*, *S. marcescens*, *Stenotrophomonas maltophila*, *Xanthobacter flavus*	*Acanthamoeba polyphaga*	(Pagnier et al. 2008)
	Candidatus "*Roseomonas massiliae*", C. "*Rhizobium massiliae*", C. "*Chryseobacterium massiliae*", and C. "*Amoebinatus massiliae*"	*Acanthamoeba polyphaga*	(Greub et al. 2004)
	Bacillus licheniformis	*Naegleria fowleri*	(Lebbadi et al. 1995)
	Staphylococcus aureus	*Acanthamoeba polyphaga*	(Huws et al. 2008)

Table 2.4 continued

Relationship of the bacterium to amoeba	Bacterium	Amoeba	Reference(s)
Intracellular survival shown in laboratory	*Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa*	*Acanthamoeba rhysodes*	(Walochnik et al. 1998)
	Pseudomonas aeruginosa	*Hartmanella cantabrigiensis*	(Walochnik et al. 1998)
	Salmonella typhimurium, Yersinia enterocolitica, Shigella sonnei, Legionella gormanii, Campylobacter jejuni, Escherichia coli, Citrobacter freundii, Enterobacter agglomerans, Enterobacter cloacae, Klebsiella pneumoniae, Klebsiella oxytoca	*Acanthamoeba castellanii*	(King et al. 1988)
	Campylobacter jejuni	*Acanthamoeba polyphaga, A. castellanii*	(Axelsson-Olsson et al. 2005; Snelling et al. 2005)
	28 species of *Mycobacterium*, e.g. *M. avium, M. abscessus, M. malmoense, M. intracellulare, M. massiliense, M. septicum, M. terrae*	*Acanthamoeba polyphaga*	(Adékambi et al. 2006; Steinert et al. 1998; Whan et al. 2006)
	Burkholderia pseudomallei	*Acanthamoeba astronyx*	(Inglis et al. 2000)
	Burkholderia cepacia complex	*Acanthamoeba polyphaga*	(Lamothe et al. 2004)
	Aeromonas sp.	*Acanthamoeba* sp.	(Hagnere and Harf 1993)
	Coxiella burnetii	*Acanthamoeba castellanii*	(La Scola and Raoult 2001)
	Candidatus "*Nordella oligomobilis*"	*Acanthamoeba polyphaga*	(La Scola et al. 2000, 2004b)
	Vibrio cholerae	*Naegleria gruberi*	(Thom et al. 1992)

The effects of co-cultivation for amoebae and bacteria

When amoebae and bacteria are growing together, changes often occur in either or both organisms. The most profound changes are likely to occur when the organisms adapt to endosymbiosis; these changes include alterations in their morphology and in the gene expression (Jeon 1995, 2004; Jeon and Jeon 2004). Furthermore, the presence of bacterial endosymbionts may increase or decrease the growth rate of the host amoeba (Collingro et al. 2004). Amoeba may also acquire properties to promote its survival in adverse conditions from the bacteria harbored intracellularly, for example an *Acanthamoeba* sp. acquired resistance to mercury from the presence of intracellular mercury-resistant *Aeromonas* sp. (Hagnere and Harf 1993).

However, even a passing interaction between amoebae and bacteria can result in modulation of the characteristics of both of the species. The best characterized of these effects is pathogenicity; intracellular growth within amoebae can enhance the pathogenic potential of bacteria thus acting as a "training ground" for the these bacteria (Molmeret et al. 2005). For example, co-cultivation with *Acanthamoeba castellanii* increases the ability of *Legionella pneumophila* to enter into human epithelial and monocytic cells and mouse macrophages *in vitro* and enhances virulence in mice (Cirillo et al. 1994; 1999). The same phenomenon has also been shown for *Mycobacterium avium* (Cirillo et al. 1997) and even for the soil fungus *Cryptococcus neoformans* (Steenbergen et al. 2003). Furthermore, bacteria may become more resistant to antimicrobial compounds while they are entombed inside amoebae (Miltner and Bermudez 2000), and this effect remain even after the co-cultivation as shown for *L. pneumophila* (Barker and Brown 1995). On the other hand, the interaction with bacteria can increase the pathogenicity of amoebae: co-cultivation with *E. coli* and *Salmonella* reactivated the virulence of an avirulent *Entamoeba histolytica* strain (Mirelman 1987). The existing virulence of another *E. histolytica* strain was further enhanced after co-cultivation with intestinal or gram-negative bacteria (Anaya-Velazquez and Padilla-Vaca 1992; Bracha and Mirelman 1984). It has also been suggested that intracellular bacteria could increase the pathogenicity of *Acanthamoebae* (Fritsche et al. 1998; Marciano-Cabral et al. 2003).

Bacteria need not be intracellular to evoke changes in the amoebae, and vice versa. For example, when the isoenzyme patterns of an *Acanthamoeba* strain were characterized, remarkable changes were observed between monoxenically (i.e. cultured with one strain of substrate microbe) and axenically (cultured without microbes as substrate) grown amoebae. Similar results were found also for a *Hartmanella* strain in the same study (Weekers and De Jonckheere 1997). Correspondingly, a decrease in the proteolytic activity of *E. histolytica* was observed after the axenic culture was reassociated with mixed bacteria (Spice and Ackers 1992). Furthermore, the presence of *Pseudomonas aeruginosa* in contact lens

solution enhanced the survival of *Acanthamoeba castellanii* (Cengiz et al. 2000). Some bacteria can produce compounds which are cytotoxic to amoebae thus preventing amoebal growth and survival (e.g. *Bacillus licheniformis* produces amoebicin that kills *Naegleria fowleri* amoebae) (Cordovilla et al. 1993). Furthermore, amoebae can stimulate and control bacterial growth possibly due to selective grazing and through the presence of extracellular metabolites produced by the amoebae, at least in the soil environment (Jjemba 2001; Rønn et al. 2002).

In general, it seems likely that interactions of any kind, be it intracellular, competitive, or antagonistic, between bacteria and amoebae and perhaps even between all organisms, causes changes and adaptations in some aspect of the organisms' functions.

2.2.3 Amoebae and intracellular microbes other than bacteria

Free-living amoebae have occasionally been observed with intracellular organisms other than bacteria. These organisms include other eukaryotes, such as protozoa, fungi and possibly microsporidians, but also viruses have been found to reside in amoebae. One of the few examples of protozoal endosytobionts is a case where a *Neoparamoeba* spp. was found to host *Perkinsiella amoebae* -like flagellate endosymbionts (Dyková et al. 2003b). The only fungal endosytobiont of amoebae described in literature is the soil fungus *Cryptococcus neoformans*, an opportunistic human pathogen encountered inside amoebae. In laboratory conditions, the fungus was able to evade lysis in amoebae, but the interaction increased the pathogenicity of the fungus towards human macrophages (Steenbergen et al. 2003). Also, microsporidian-like parasites have been detected in amoebae isolated from both clinical and environmental samples (Hoffmann et al. 1998; Michel et al. 2000). One further example of eukaryotic endosytobionts in amoebae are algae. A marine amoeba was found to be able to photosynthesize, due to a symbiotic relationship with an alga which occurred early in the evolution of the amoeba (McFadden et al. 1994).

Some information is available of the viruses present inside amoebae, although they are rarely studied and are sometimes stumbled upon by accident. For example, an amoebal co-culture was used to isolate bacteria associated with a pneumonia outbreak. One of the organisms isolated was first classified as bacteria, but subsequent studies revealed it to be a giant icosahedral DNA virus (La Scola et al. 2003; Raoult et al. 2007). The virus was named *Mimivirus* for mimicking microbes, and it is the largest known virus both in terms of size (0.8 µm) and genome length

(1.2 Mb). A few studies have been conducted on human adenoviruses, where 236 *Acanthamoeba* strains from the Canary Islands were screened (Lorenzo-Morales et al. 2007). As many as 34 strains (14 %) were carriers of adenoviruses indicating that amoebae could be important environmental reservoirs of adenoviral diseases. Furthermore, laboratory experiments revealed that acanthamoebae are potential promoters for the survival and transmission of coxsackie B3 viruses (Mattana et al. 2006). In contrast, acanthamoebae served only as solid-like carriers of echoviruses and polioviruses with no intracellular replication being observed under laboratory conditions (Danes and Cerva 1981).

These studies indicate that the interaction with amoebae is not limited to bacteria, but many kinds of microbial organisms intermingle with amoebae. Very little is known of the consequences of these interactions and further studies are needed to determine the role of amoebae in association with these organisms.

2.2.4 Identification methods of environmental amoebae

The free-living amoebae have traditionally been identified according to their morphological characteristics under light microscopy and sometimes by electron microscopy. Even today, when molecular methods are becoming more and more readily available, morphological typing is a helpful tool in distinguishing groups of amoebae from each other. Genera and even some families defined by morphological characteristics have been found to form clusters in molecular trees (Fahrni et al. 2003), although typing to species level is often erratic if one must rely only on morphology. Tests on enflagellation, encystation, salinity tolerance and other physiological characteristics can be used in conjunction with morphological typing to aid the identification (Smirnov and Brown 2004). Several identification guides (e.g. Lee et al. 2000; Page 1988; Smirnov and Brown 2004) have been published. All of these offer slightly different classification systems for amoebae, and should be used as practical guide to morphotypes rather than for taxonomical grouping.

Before any morphotyping of the amoebae can be made, the amoebae need to be isolated or cultured out of the environmental or clinical sample. Only a fraction of the total number of amoebae can be seen directly from the sample, because they are often flattened and attached to soil and other particles (Smirnov and Brown 2004). The culturing and subsequent cloning of the amoebal strains may require weeks of time if one wishes to determine the fauna in environmental samples, because a succession of species may need to thrive at different times before the inactive amoebae in the samples can be detected. Due to the limitations of morphological

techniques, molecular methods for identifying amoebae directly from the samples are constantly being developed.

At the moment, there are hundreds of submissions in the Genbank for amoebal sequences, mostly for ribosomal RNA-coding DNA, but also for mitochondrial 16S DNA. This has allowed the design of many genus- and species-specific primers and amplimers, e.g. for the genera *Acanthamoeba* (Schroeder et al. 2001; Vodkin et al. 1992), *Naegleria* (Schild et al. 2007), the species *Hartmanella vermiformis* (Kuiper et al. 2006) and *Balamuthia mandrillaris* (Booton et al. 2003). Attempts have been made in order to produce a primer pair that would be able to detect all free-living amoebae (Tsvetkova et al. 2004), but this has proved non-specific and amplification of *Tetrahymena pyriformis*, a ciliated protozoan, was also observed with this primer design (Behets et al. 2007). Even quantitative real-time PCR has been developed for some groups, e.g. for acanthamoebae (Rivière et al. 2006), and to permit the simultaneous detection of the three pathogenic amoebae (acanthamoebae, balamuthia, and naegleriae) from clinical samples (Qvarnström et al. 2006). Many of these methods do not require prior culturing of the amoebal strains, instead, the amoebae can be detected directly from the sample material. This field of amoebology is developing rapidly and more sensitive and specific methods are likely to become available within the near future.

To summarize the literature cited in this review, amoebae are an important part of microbial ecosystems everywhere. They have a central role in regulating and modulating the characteristics of bacteria and probably other microbes with which they interact, possibly altering also the human health effects associated with these microbes. Elaborate microbial ecosystems gradually develop on damp materials present in moisture-damaged buildings, and it is reasonable to assume that amoebae will be one part of these microbial networks. However, very little is known of the amoebae possibly occurring in moisture-damaged buildings and perhaps ending up in the indoor environment. The current study was conducted to address this problem, and to elucidate the role of amoebae in moisture-damaged buildings.

3 AIMS OF THE STUDY

The overall aim of the study was to elucidate the role of amoebae in the microbial network in moisture-damaged buildings, and thus as one of the agents contributing to the exposure and health effects observed in this type of environment. The specific aims were:

1. To investigate the occurrence of amoebae in moistened material samples from moisture-damaged buildings, to determine whether the occurrence of amoebae is associated with the presence of other microbes, and to evaluate whether amoebae can be used as indicators of moisture damage in a building (I).

2. To determine the ability of *Acanthamoeba polyphaga* to grow on different building materials (II).

3. To observe the effects of *Acanthamoeba polyphaga* on the growth and viability of selected microbes isolated from moisture-damaged buildings (III).

4. To evaluate whether *Acanthamoeba polyphaga* can exert effects on the cytotoxic and proinflammatory potential of selected bacteria and fungi isolated from moisture-damaged buildings (IV).

4 MATERIALS AND METHODS

4.1 Overall study design

This research comprises of a set of four studies designed to illustrate the role of amoebae as potential exposing agents in moisture-damaged buildings. First, the occurrence of amoebae in moisture-damaged buildings was examined by screening damaged building material samples for amoebae. The samples were also cultivated for fungi and bacteria and the connection between the incidence of fungal species and amoebae was studied (Study I). Since the occurrence of amoebae on the building material samples was not homogenous within the various building materials, the ability of amoebae to survive on different sterilized building materials both used and unused was then investigated under laboratory conditions (Study II). Thirdly, the effects of amoebae on the growth and viability of certain fungi and bacteria isolated from moisture-damaged buildings, and vice versa, were examined by co-cultivation in the laboratory (Study III). Finally, the changes in the proinflammatory and cytotoxic potential of these microbes caused by the co-cultivation were tested by exposing RAW264.7 mouse macrophages in a dose-dependent manner to individually grown and co-cultured samples of the microbes and by measuring the subsequent cytotoxicity and production of inflammatory markers such as NO and proinflammatory cytokines (Study IV). The methods of each study are described briefly in this section, with the detailed procedures being presented in the original publications.

4.2 The occurrence of amoebae in building material samples from moisture-damaged buildings (I)

The presence of amoebae was determined from 124 building material samples taken from moisture-damaged buildings. The quantity of the amoebal cells in the samples was then approximated. The same samples were also cultivated for fungi and bacteria and of these the fungi were identified to the genus level. Actinomycete counts were recorded separately based on their colony morphology. Finally, the water content of the material samples was determined. The amoebal occurrence and abundance was compared against the bacterial and fungal types and their cfu-counts, and against the water content in the samples.

4.2.1 Sampling of building materials (I)

Building material samples (n = 125) were taken from Finnish office buildings, schools and family residences to screen for the occurrence of amoebae. The samples were taken directly from the building structure with sterilized tools and inserted into plastic bags. All the samples were taken from buildings suspected to be moisture-damaged and samples were taken from several locations in the building. The samples were usually taken during a technical investigation of the building for possible mold and moisture damage.

4.2.2 Detection and quantification of amoebae from building material samples (I)

In this study, amoebae were detected and their numbers were roughly estimated from samples taken from moisture-damaged buildings. Amoebae were detected from the samples by the method adapted from Newsome et al. (1998). In this method, two stripes, an "X", of heat-killed *Escherichia coli* (ATCC 25922) were streaked on non-nutritive agar plates. A piece of the sample (ca. 1×1 cm^2) was placed in the center of the *E. coli* X on the plate and the plates were incubated at 25 °C for 48 - 72 hours. Plates were examined microscopically along the *E. coli* lines and around the material sample at magnifications of 100 to 400 for the presence of amoebae. The amounts of trophozoites and cysts on each plate were estimated with a five-step log-scale classification (not present = 0, up to 10 trophozoites and cysts = 1, up to 100 = 2, up to 1000 = 3, more than 1000 = 4).

4.2.3 Detection and identification of fungi and bacteria from the building material samples (I)

The building material samples were cultivated for fungi and bacteria either by direct plating (n = 75) or by dilution plating (n = 49) (Hyvärinen et al. 2002; Reiman et al. 1999). In the direct plating, approximately 0.5 ml of the homogenized material sample was dispersed evenly on four types of agar plates: fungi were grown on Rose Bengal malt extract agar (Hagem), dichloran glycerol agar (DG18) and 2 % malt extract agar (MEA), and bacteria on tryptone yeast extract glucose agar (TYG) (Reiman et al. 1999). In the dilution plating, samples were weighed (1-5 g) and

extracted with dilution buffer, sonicated, and shaken for 30 and 60 minutes respectively. Aliquots of 100 µl of serial dilutions were spread evenly on MEA, DG18 and TYG -media (Hyvärinen et al. 2002). All samples were incubated in the dark for 5 days (bacteria), 7 days (fungi) or 14 days (actinomycetes) at 20-25 °C. The numbers of fungal colonies were counted on plates after the incubation and calculated as colony forming units (cfu) per gram of material. Fungi were identified to the genus level by light microscopy; however, *Aspergillus versicolor*, *A. fumigatus*, *A. terreus* and *A. niger* were identified to species level.

4.3 The survival of amoebae on building materials (II)

The survival of amoebae on various building materials was determined under laboratory conditions. A variety of building materials, both unused and used, were obtained either from buildings undergoing a renovation (old materials) or bought from a hardware store (unused materials). A total of six material types were tested; of these both unused and old versions were tested for four materials. The old materials were selected so that no visible microbial growth was present and the materials were sterilized by autoclaving prior to the start of the experiment. The materials used for the amoebal growth study were:

- Concrete, unused and old
- Linoleum, unused and old
- Mineral insulation, unused and old
- Pine wood, unused and old
- Birch wood, unused
- Gypsum board, unused

The materials were cut to pieces of app. 1 cm × 1 cm of size, which were thoroughly wetted with sterile spring water. Then, half of the number of the samples were smeared with bacterial water suspension prepared from heat-killed *E. coli*. The materials were wetted in order to provide a water film for amoebae and the *E. coli* suspension served as additional nutrient source. Finally, samples were inoculated with 20-50 µl of amoebal suspension containing 1000 amoebae. The samples were then placed on sterile Petri dishes in air-tight glass containers. A layer of sterile water was put on the bottom of the container in order to ensure a relative humidity

of 100 % in the container. The samples were not in direct contact with the water. To avoid anaerobic conditions, the containers were aerated daily with filtered air. The samples were incubated in the containers for either 0, 2, 7, 14, or 56 days. Twelve replicate samples were prepared for each incubation endpoint, six of which were treated with *E. coli* and six were without *E. coli*. The presence of amoebae from the samples was determined as in the study I but only the presence/absence of the amoebae was included in the statistical analyses.

4.4 The effects of co-cultivation of fungi and bacteria with amoebae on their growth, viability, and immunotoxic potential (III, IV)

In order to investigate whether the occurrence of amoebae in moisture-damaged buildings has any effect on the fungi and bacteria also present, amoebae and selected fungi and bacteria were co-cultivated under laboratory conditions. Known concentrations of amoebae were incubated with known concentrations of bacterial cells or fungal spores in water suspension, and the changes in the concentrations (both viable and total concentration) were followed periodically from 0 to 28 days. After the co-cultivation, samples from certain time points were selected for toxicological investigations. In these tests, the mouse macrophage cell line (RAW 264.7) was exposed dose-dependently to separately grown fungi, bacteria and amoebae, and also exposed to co-cultivations of fungi and amoebae, and bacteria and amoebae. The cytotoxicity of these combinations were tested along with their ability to induce the production of NO and proinflammatory cytokines, interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α). The amoebal, fungal and bacterial strains selected are described in Table 4.1. The macrophage cell line RAW264.7 used in study IV was obtained from American Type Culture Collection (strain TIB-71) and it originated from ascites tissue.

Table 4.1 The microbial strains used, their sources and identification authorities

Strain	Description	Source	Identification	Study
Acanthamoeba polyphaga	Ubiquitous opportunistic free-living environmental amoeba, able to form cysts	ATCC strain 30461, originally from eye infection	ATCC[1]	II, III, IV
Stachybotrys chartarum	Potentially toxin-producing fungus, indicates unusual indoor microbial source	Building material sample from a moisture-damaged building, Finland	CBS[2]	III, IV
Aspergillus versicolor	Potentially toxin-producing fungus, indicates unusual indoor microbial source	Indoor air of moisture-damaged building, Finland	CBS[2]	III, IV
Penicillium spinulosum	Potentially toxin-producing fungus, representative of indoor air penicillia	Indoor air of moisture-damaged building, Finland	CBS[2]	III, IV
Streptomyces californicus	Potentially toxin-producing actinobacterium	Indoor air of moisture-damaged building, Finland	DSMZ[3]	III, IV
Bacillus cereus	Potentially toxin-producing gram-positive bacterium common in the environment and indoor air	Indoor air of moisture-damaged building, Finland	Evira Kuopio[4]	III
Pseudomonas fluorescens	Gram-negative bacterium	Indoor air of a building, Finland	Evira Kuopio[4]	III, IV

[1]American Type Culture Collection, Manassas, Virginia, USA; [2]Centraal Bureau of Schimmelcultures, Utrecht, the Netherlands; [3]Deutsche Sammlung von Microorganismen and Zellkulturen, Germany; [4]Finnish Food Safety Authority, Kuopio Regional Laboratory, Finland

4.4.1 Upkeep and preparation of microbial cultures

Amoebae (II, III, IV)

Acanthamoeba polyphaga amoebae were initially stored in liquid nitrogen and then grown in peptone yeast glucose (PYG) broth at 25 ºC. The culture was renewed weekly by transferring a portion of the suspension to fresh PYG growth medium until the active growth phase was reached in about 10 weeks after thawing. A new lot of frozen amoebae was activated for each experiment.

In the experiments, approximately 10^5 amoebae in 0.25 ml of suspension were added into 5 ml of PYG broth and grown at 25 °C in tissue culture flasks. Several replicate flasks were prepared at the same time. After 7 days of incubation, the PYG broth was carefully removed to leave a layer of amoebae on the bottom of the flask. The layer of amoebae was then washed once with 10 ml of sterile spring water, the water was removed, and the cells were finally suspended into a fresh 5 ml aliquots of sterile spring water.

All of the replicates were pooled and the concentration of amoebae in the water suspension was assessed in a Bürker chamber with Trypan-blue vital stain. Both viable and total concentrations were recorded. The concentrations needed for the growth experiments were adjusted according to the viable counts. Depending on the experiment, the suspension was used as such, diluted, or concentrated by centrifugation to the desired amoebal concentration.

Bacteria and fungi (III, IV)

The bacterial and fungal strains were cultured on agar media (tryptone yeast extract glucose agar (TYG) for bacteria and 2 % malt extract agar (MEA) for fungi) and grown at 25 °C for 7 days. Microbial cells or spores were then collected with a sterile 10 µl plastic loop and placed into 5 ml of sterile spring water. Finally, the concentrations of bacteria in the suspensions were analyzed in duplicate by staining with 0.01 % acridine orange and direct counting (AODC-method) with an epifluorescence microscope. Two hundred bacterial cells or spores or a maximum of twenty fields were counted. The concentrations of the fungal spores in the suspension were counted in duplicate using a Bürker chamber.

4.4.2 *In vitro* studies (IV)

Cell culture of RAW264.7 macrophages

Mouse RAW264.7 macrophages were cultured in RPMI 1640-medium including 10 % heat-inactivated fetal bovine serum, 1 % l-glutamine and 1 % penicillin-streptomycin. Cells were dispensed in a concentration of 5×10^5 cells ml^{-1} into 6-well plates and allowed to adhere for 24 hours before the exposures. Fresh complete medium was exchanged 1 h before the exposure.

Exposure

Macrophages were exposed for 24 hours to 1) separately grown amoebae, dose 1000 ml^{-1}, 2) separately grown fungi and bacteria, doses 3×10^4, 10^5, and 3×10^5 ml^{-1}, and 3) to co-cultures of each fungal and bacterial strain with the amoebae in the same doses as above.

Cytotoxicity analysis

After 24-h exposure, the viability of the macrophages was measured with the MTT test, a traditional cytotoxicity test (Mosmann 1983). Live cells transform exogenously administered MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] to the colored formazan via their intact mitochondria. Formazan can in turn be measured spectrophotometrically at a wavelength of 570 nm. The proportion of viable macrophages in the exposed samples was reported as a percentage of viable cells when compared to cells in HBSS control samples.

Inflammation analyses

Nitric oxide analysis

NO was measured spectrophotometrically in the culture medium as the stable metabolite, nitrite (NO_2) using the Griess method (Green et al. 1982). Griess reagent (1 % sulphanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2 % phosphoric acid) was mixed 1:1 with samples of the fresh cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at a wavelength of 543 nm, which was measured using a microplate reader. The

nitrite concentrations were calculated by comparing the absorbance of the samples with those of standard solutions of sodium nitrite.

Cytokine analysis

The cytokine analyses (TNFα, IL-6) were performed with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. The samples were analyzed with a microplate reader at a wavelength of 450 nm. Cytokine concentrations of samples were calculated by interpolating absorbances of samples to the standard curve.

4.5 Statistical analyses

All statistical analyses were performed with SPSS (versions 10.1 to 12.). In study I, the associations between the presence of amoebae and the presence of fungal genera were compared with Fishers' test, and the comparisons between the abundance of fungal and bacterial growth with the abundance of bacteria were performed with Mann-Whitney test. In study II, the effects of the *E. coli* treatment, the age of the material, and incubation time on amoebal survival were tested with crosstabulation and Fisher's or Chi-square tests. The amoebal preference ranking of the materials was created by calculating the percentage of amoebal survival on each material; the material with the highest survival percentage was considered preferred by amoebae. In study III, the differences between the microbial counts in co-cultured and individually grown samples were compared with the linear mixed model for multivariate repeated samples, because the samples taken at the incubation endpoints were subsamples from the same growth suspension. In study IV, the comparisons were made with ANOVA, as the incubation endpoints were all prepared separately and thus they were independent from each other.

5 RESULTS

5.1 The occurrence of amoebae in building material samples from moisture-damaged buildings (I)

Amoebae were detected in 27 (22 %) building material samples out of the total of 124 samples studied (I). The samples in which the amoebae were found were usually clearly damaged with abundant microbial growth. Amoebae were never detected on building material samples without other microbial growth, especially bacteria. Bacteria were always present in the samples with amoebae, but fungi were not necessarily detected in these samples. In this study, both fungi and bacteria were simultaneously found in most samples (86 %). Only 8 % of the samples exhibited fungal growth but no bacteria; amoebae were not present in these samples. Even less (3 %) of the samples had only bacteria, but no fungi. Amoebae were detected on some of these samples. In the building material sample database of over 1300 samples in the National Institute for Health and Welfare (former Public Health Institute), it is stated that culturable fungi are present in approximately 72 % and culturable bacteria in 85 % of the samples (unpublished data).

A statistically significant co-occurrence with amoebae was found for the fungi *Acremonium* ($p<0.0081$), *Aspergillus versicolor* ($p<0.0232$), *Chaetomium* ($p<0.0231$), *Trichoderma* ($p<0.0043$), and for bacteria belonging to the group actinomycetes ($p<0.0004$), as well as for all bacteria in general ($p<0.041$). These relationships are illustrated in Figure 5.1. Furthermore, when comparing the amoebal and fungal/bacterial numbers, the higher the counts of these microbes (except *Trichoderma*) and Sphaeropsidales, *Stachybotrys*, and *Paecilomyces* were, the higher the counts of amoebae also detected.

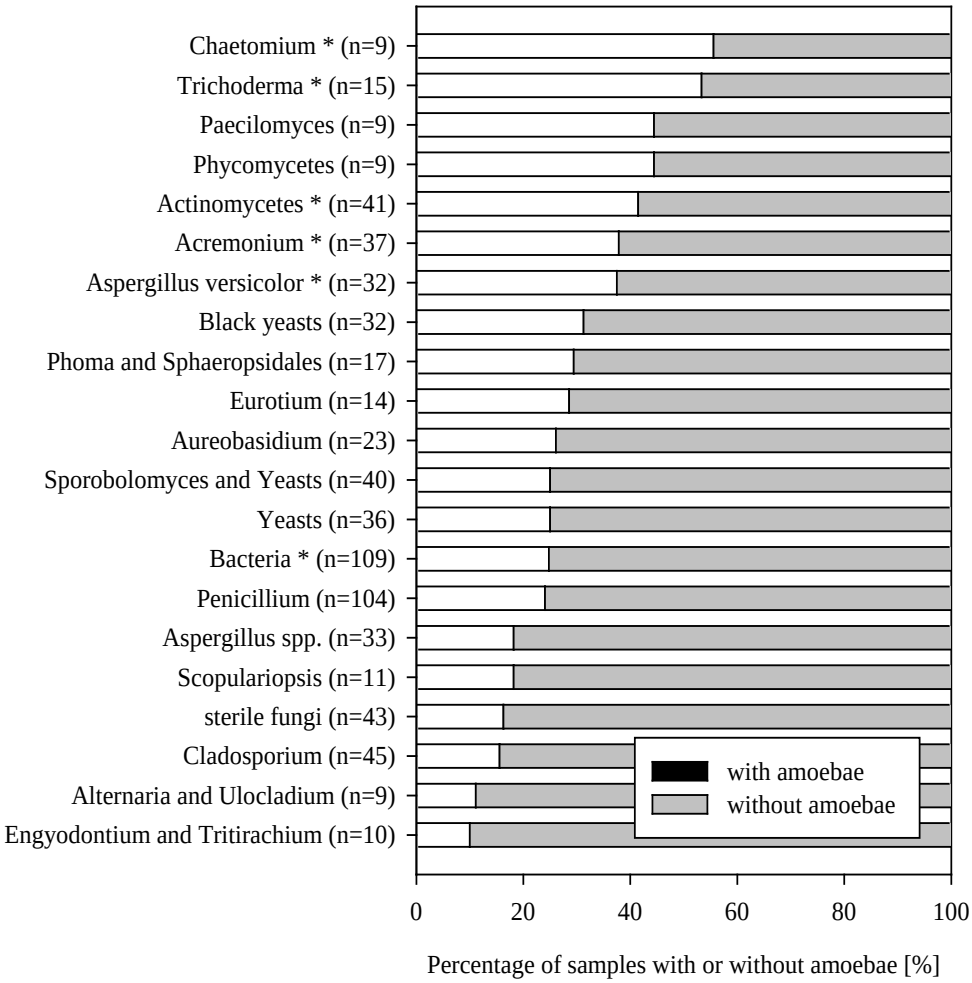

Figure 5.1 The percentage of microbial genera or groups occurring with or without amoebae. An asterisk denotes a statistically significant co-occurrence with amoebae (modified from Yli-Pirilä et al., 2004)

Furthermore, the water content (w/w percentage) of the samples with amoebae was on average higher than that of the samples without amoebae (31 ± 11 % vs. 3.5 ± 0.6 %, respectively, $p<0.0001$), and amoebae seemed to be more abundant when there were higher water contents. However, amoebae were also found in a sample with a water content as low as 0.4 % (mineral insulation sample). In addition to the moisture content, the type of the building material also affected the occurrence of

amoebae. Amoebae were most often found on wood samples, and least often on mineral insulation samples (38 % and 8 % of samples were positive for amoebae, respectively). On the other hand, the water contents of these and other materials varied significantly, with wood samples being the wettest.

Even though most building material samples were taken from a building with moisture damage in at least one area, not all individual samples were damaged. Every sample analyzed had also been given an individual status as to whether the sample was moisture-damaged or not based on the fungal and actinomycete concentrations and fungal species present on the sample. A total of 80 % of the samples were considered damaged, whereas 20 % of the samples were non-damaged. Amoebae were detected almost exclusively on the damaged samples, and not on the non-damaged materials. As many as 24 % of the analyzed samples deemed moisture-damaged sported also amoebae. Amoebae were found only on one non-damaged building material sample, and in two samples of sand and moss which were not considered damaged but neither of these can be considered as building materials as such. In the statistical comparison, amoebae occurred significantly more often on the damaged than non-damaged building material samples ($p<0.019$), if the samples of sand and moss were excluded from the calculations.

5.2 Survival of amoebae on different building materials (II)

The survival of amoebae varied extensively on the building materials studied in the experimental setting. Generally, the highest percentage of survival of amoebae was found on gypsum board and birch wood, whereas no amoebae were detected on fresh pine. The rank order of the materials in relation to amoebal survival on the samples was roughly as follows: gypsum board, birch wood and old pine wood, mineral insulation, linoleum, concrete, and finally unused pine wood.

Furthermore, the availability of nutrients and the length of the incubation period also affected the amoebal survival on the material samples. For all of the materials, nutrient supplementation increased the percentage of samples positive for amoebae throughout the incubation study, although the increase was statistically significant only for old pine ($p<0.0001$) and birch wood ($p<0.013$). On the other hand, the respective percentages decreased steadily during the incubation period on the samples without *E. coli*, whereas on the samples treated with *E. coli* no such trend was so clearly evident. These trends are illustrated in Figure 5.2. Instead, on *E. coli* -treated samples there seemed to be a sort of on-off situation: either amoebae were

present on almost 100 % of the samples or they had disappeared entirely. Amoebal survival was observed on the occasional sample of concrete but these samples consisted mostly of stone pebbles imbedded in the material.

The ageing of the building material had little effect on the survival of amoebae with the exception of pine wood. Pine wood differed from the other materials studied as the amoebae did not survive at all on fresh wood whereas old pine wood proved to be a good support material for the amoebae until the end of two-month incubation period, as long as *E. coli* had been provided ($p<0.0006$). These kinds of drastic differences in the amoebal survival were not observed for the other materials.

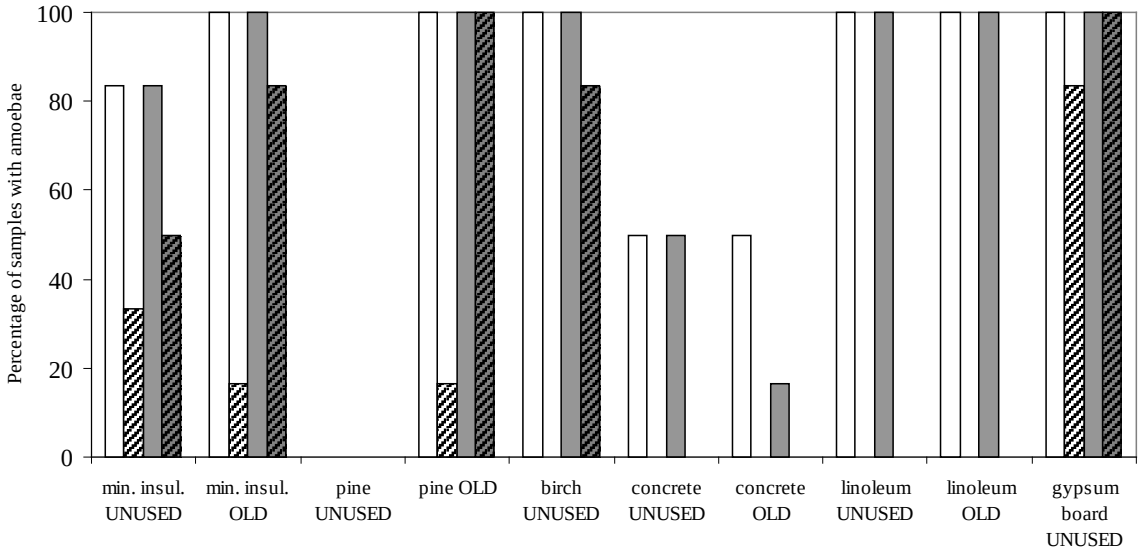

Figure 5.2 The percentage of material samples positive for amoebae at the beginning of the experiment (solid bars) and after 2 months' incubation (lined bars). Samples without *E. coli* supplementation are white and with *E. coli* grey (modified from Yli-Pirilä et al., 2009)

5.3 The effects of co-cultivation of amoebae with fungi or bacteria on their growth and viability (III)

There were significant differences in the fungal, bacterial and amoebal counts between the co-cultivated and the individually grown samples, as shown in Figures 5.3, 5.4 and 5.5. However, the changes in the fungal and bacterial numbers varied depending on the species being cultured. Generally, the bacterial counts reached higher levels in the company of amoebae than on their own. In the co-cultures, the bacteria *Pseudomonas fluorescens* and *Bacillus cereus* exhibited a rapid response to the presence of amoebae by showing a higher growth peak already at day 1 ($p<0.001$ for total counts for both species and viable counts for *P. fluorescens*; $p<0.052$ for viable counts for *B. cereus*), whereas the highest bacterial counts of *Streptomyces californicus* were found only after day 7 ($p<0.001$). Conversely, compared to the samples of individually grown amoebae, the numbers of amoebae decreased towards the end of the experiment in co-cultures with the bacteria *S. californicus* and *B. cereus* ($p<0.001$-0.038). In co-cultures with the bacterium *P. fluorescens*, there were both slightly higher and slightly lower counts than in the separately grown samples.

Unlike bacteria, no general trend was found in the differences of fungal numbers between the co-cultivated and individually grown samples, but all species behaved in a different manner. For the fungus *Stachybotrys chartarum*, numbers of viable fungi were lower in the co-cultures than in individually grown samples at the 2nd ($p<0.002$) and 3rd ($p<0.007$) week of incubation, but no differences were observed in the fungal numbers at the end of the experiment at 28 days. With the fungus *Aspergillus versicolor*, the total fungal counts were slightly higher in the co-cultures than in the individually grown samples during the first three days of the incubation ($p<0.003$, $p<0.049$, $p<0.018$, respectively), but no differences were observed thereafter or in the viable counts of the fungus. Finally, both the viable and total counts of *Penicillium spinulosum* were significantly higher in the presence of amoebae throughout the whole incubation period ($p<0.001$-0.015). Simultaneously, the amoebal counts remained more or less at the same level in both co-cultures and in controls; lower amoebal counts were observed only in co-culture with *S. chartarum* at days 1, 3, 7 and for viable counts also on day 28 ($p<0.001$-0.013).

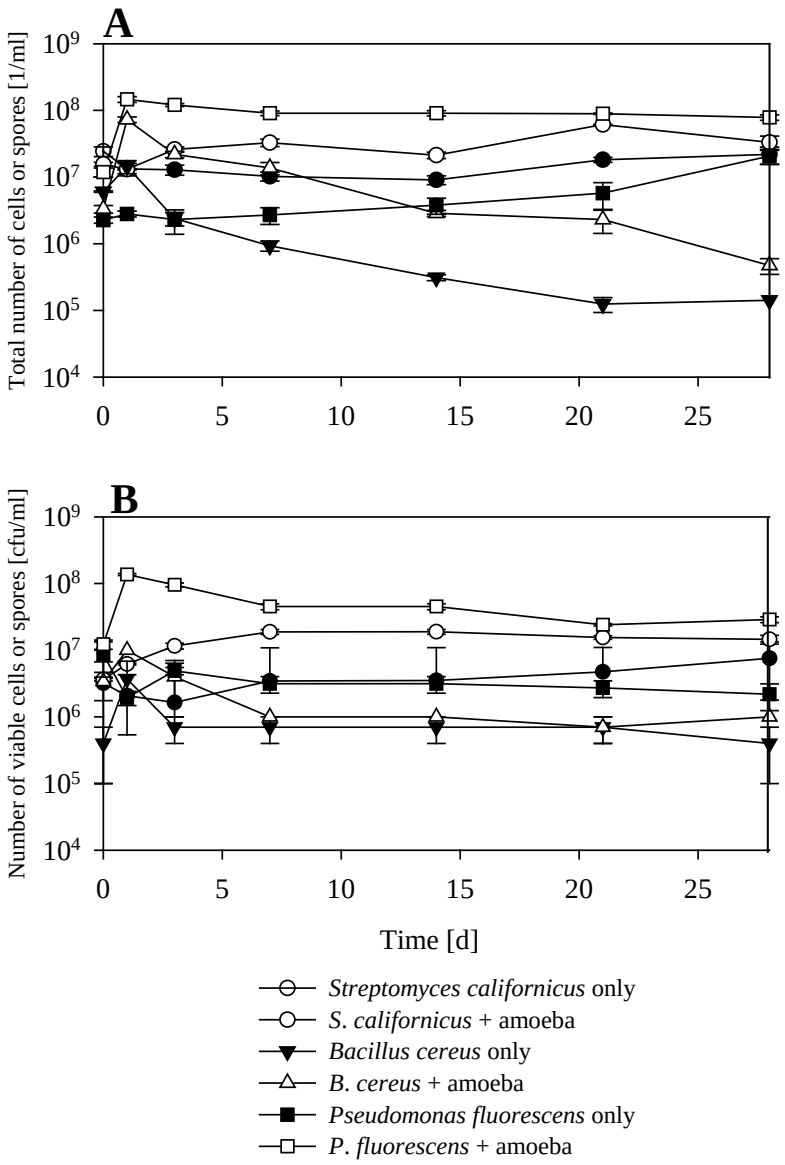

Figure 5.3 Bacterial total counts (A) and viable counts (B) in samples cultivated separately or cultivated with amoebae (modified from Yli-Pirilä et al., 2006)

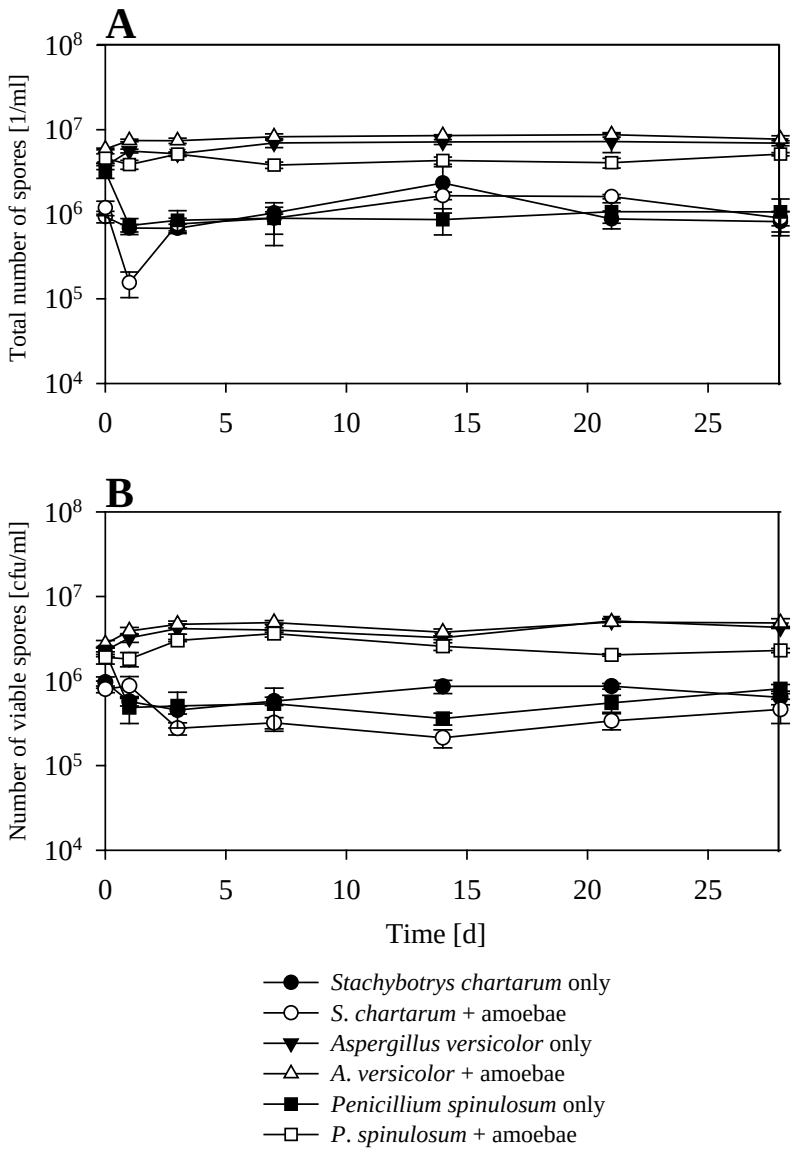

Figure 5.4 Fungal total counts (A) and viable counts (B) in samples cultivated separately or cultivated with amoebae (modified from Yli-Pirilä et al., 2006)

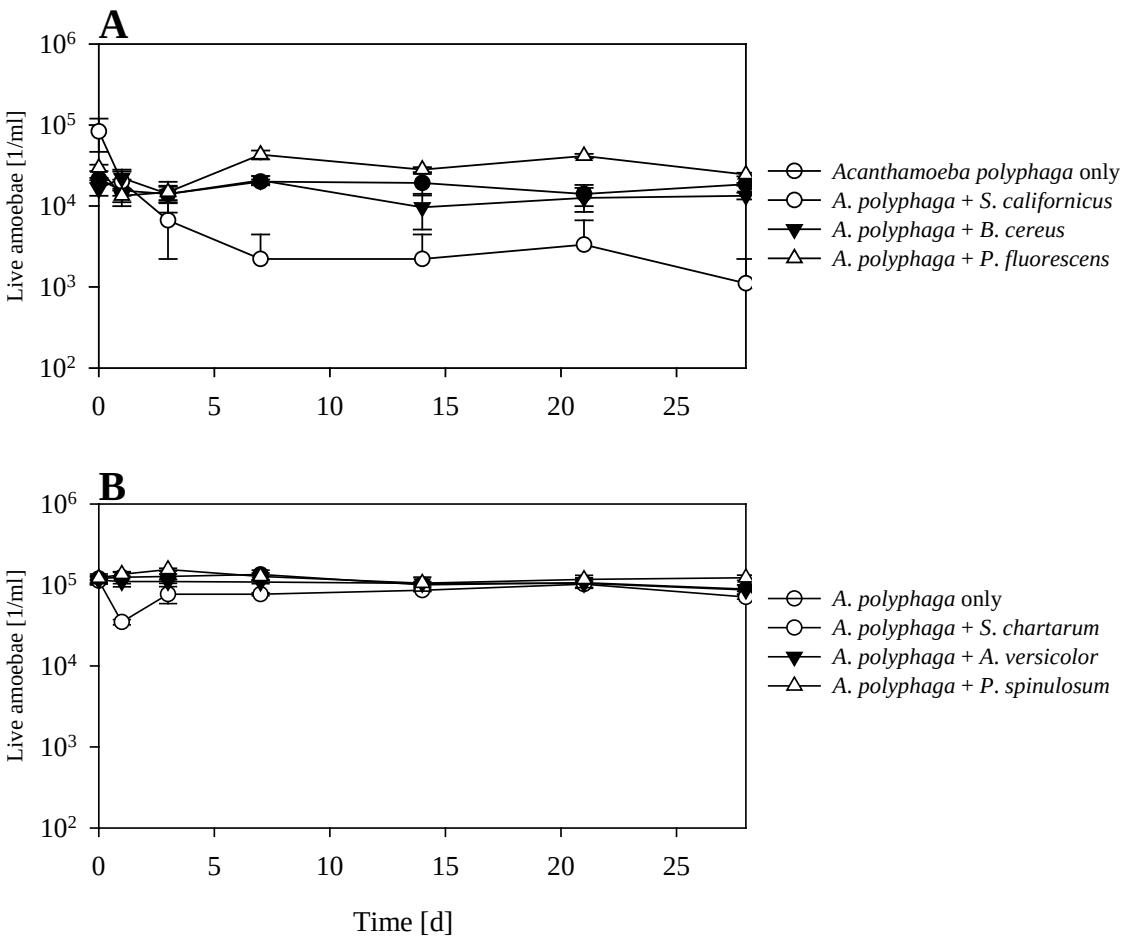

Figure 5.5 Number of viable amoebae with bacteria (A) and with fungi (B) (modified from Yli-Pirilä et al., 2006)

5.4 The effects of co-cultivation with amoebae on cytotoxicity and proinflammatory potential of microbes (IV)

The co-cultivation with amoebae increased the cytotoxicity and proinflammatory potential of two of the five studied microbes, i.e. the bacterium *Streptomyces californicus* and the fungus *Penicillium spinulosum* (Tables 5.1, 5.2). The effects were evident after different periods of co-incubation: the increase in cytotoxicity and in induced concentrations of NO, IL-6, and TNF-α appeared on day 1 of the incubation with *S. californicus* and decreased to the control level thereafter. In contrast, the effects of *P. spinulosum* could be statistically significantly seen only on day 28 at the end of the trial. The effects were dose-dependent and consistent for all markers studied. The other microbes and amoebae alone in the concentrations used were not significantly cytotoxic nor did they significantly induce the cytokine production.

Table 5.1 Cytotoxicity of samples, measured as percentage of live cells in HBSS buffer, after exposure to amoebae, amoeba-co-cultivated bacteria and fungi, and individually cultivated bacteria and fungi. Values in **bold** represent a statistically significant difference to the corresponding separately grown sample

exposure to:	culture	cytotoxicity [% of live cells in HBSS buffer] day 1	day 7	day 28
Acanthamoeba polyphaga[1]	alone	100 ± 6	96 ± 7	96 ± 12
Streptomyces californicus[2]	alone	97 ± 5	101 ± 3	116 ± 2
	in co-culture[3]	**64 ± 9** $p<0.001$	95 ± 6	100 ± 5
Pseudomonas fluorescens[2]	alone	118 ± 7	118 ± 4	117 ± 5
	in co-culture[3]	111 ± 7	114 ± 7	112 ± 10
Stachybotrys chartarum[2]	alone	94 ± 7	98 ± 12	82 ± 12
	in co-culture[3]	80 ± 14	86 ± 9	85 ± 10
Aspergillus versicolor[2]	alone	85 ± 12	88 ± 9	85 ± 8
	in co-culture[3]	78 ± 8	89 ± 5	77 ± 0
Penicillium spinulosum[2]	alone	73 ± 6	88 ± 14	77 ± 9
	in co-culture[3]	82 ± 8	76 ± 4	**52 ± 3** $p<0.008$

[1]dose 1000 ml^{-1}; [2]dose 3×10^5 ml^{-1}; [3]In co-culture with *A. polyphaga*

Table 5.2 The cytokine and NO concentrations in growth media of RAW264.7 macrophages after exposure to amoebae, amoeba-co-cultivated bacteria and fungi, and individually cultivated bacteria and fungi. Values in **bold** represent a statistically significant difference in concentration to the corresponding separately grown sample

exposure to:	culture	TNF-α [pg/ml]			IL-6 [pg/ml]			NO [µg]		
		day 1	day 7	day 28	day 1	day 7	day 28	day 1	day 7	day 28
Acanthamoeba polyphaga[1]	alone	96 ± 11	102 ± 17	106 ± 22	n. d.	n. d.	n. d.	1.1 ± 0.0	1.1 ± 0.2	1.4 ± 0.2
Streptomyces californicus[2]	alone	2134 ± 295	2503 ± 675	1332 ± 84	51 ± 39	54 ± 27	59 ± 12	2.1 ± 0.4	1.8 ± 0.1	1.6 ± 0.3
	in co-culture[3]	**7728 ± 2678** $p<0.004$	2458 ± 778	1112 ± 226	**1557 ± 311** $p<0.001$	47 ± 29	32 ± 22	**15.9 ± 3.2** $p<0.001$	2.8 ± 0.6	1.4 ± 0.2
Pseudomonas fluorescens[2]	alone	2744 ± 935	1192 ± 228	1628 ± 125	103 ± 31	37 ± 10	69 ± 27	3.1 ± 0.6	1.4 ± 0.1	1.8 ± 0.0
	in co-culture[3]	2068 ± 269	701 ± 222	1301 ± 89	85 ± 22	18 ± 3	93 ± 7	4.4 ± 1.3	1.3 ± 0.2	1.5 ± 0.2
Stachybotrys chartarum[2]	alone	745 ± 43	496 ± 104	867 ± 299	n. d.	n. d.	n. d.	1.4 ± 0.3	1.7 ± 0.4	1.5 ± 0.3
	in co-culture[3]	1341 ± 234	527 ± 77	478 ± 74	n. d.	n. d.	n. d.	1.8 ± 0.3	1.4 ± 0.2	2.1 ± 0.8
Aspergillus versicolor[2]	alone	481 ± 30	178 ± 16	201 ± 44	n. d.	n. d.	n. d.	1.8 ± 0.3	1.6 ± 0.3	1.6 ± 0.1
	in co-culture[3]	503 ± 14	275 ± 52	**369 ± 47** $p<0.009$	n. d.	n. d.	n. d.	1.3 ± 0.3	1.3 ± 0.4	1.2 ± 0.2
Penicillium spinulosum[2]	alone	113 ± 28	100 ± 17	168 ± 10	n. d.	n. d.	2 ± 2	1.2 ± 0.3	1.2 ± 0.2	1.3 ± 0.3
	in co-culture[3]	137 ± 11	**286 ± 69** $p<0.002$	**4180 ± 898** $p<0.001$	n. d.	n. d.	**396 ± 68** $p<0.001$	1.5 ± 0.4	1.5 ± 0.6	**14.4 ± 1.4** $p<0.001$

[1]dose 1000 ml^{-1}; [2]dose 3×10^5 ml^{-1}; [3]In co-culture with *A. polyphaga*; n.d. = below detection limit

6 DISCUSSION

6.1 The role of amoebae as members of microbial network in moisture damage

Amoebae are ubiquitous organisms that can survive in most environments containing water. The prevalence of amoebae has been mainly studied in natural environments. With respect to environments where they are in close proximity to humans, amoebal investigations have been conducted in swimming pools (e.g. Rivera et al. 1993; Vesaluoma et al. 1995), drinking water systems (e.g. Hoffmann and Michel 2001; Michel et al. 1995a) and in hospitals (e.g. Rohr et al. 1998). Even though moist building materials and other moist locations in buildings could offer sites for amoebal survival, there is limited knowledge of the amoebal occurrence in buildings.

The results of this study demonstrate that amoebae do indeed live on moisture-damaged building materials. Amoebae were detected in approximately every fifth building material sample taken from moisture-damaged buildings. Most of these samples were considered as damaged judging by their fungal and bacterial concentrations and fungal genera, but 20 % of the samples were considered non-damaged. Interestingly, amoebae were almost exclusively found on the samples judged as damaged. Correspondingly, when statistically comparing prevalences of microbial species, amoebae occurred often together with the fungi and bacteria that are considered as indicators of moisture damage in buildings.

The samples positive for amoebae had on average a higher moisture content than samples without amoebae. Furthermore, the more moist the samples were, the more abundant were the rough estimations of amoebal numbers. It is known that in other environments amoebae require free water to move and reproduce, although an aqueous layer of few micrometers will suffice (Hausmann et al. 2003). These microscopic layers could easily occur on surfaces or in pores of wet building materials, providing niches for amoebal growth. However, in the current study amoebae were also found in a few samples where the absolute water content was less than 1 %. These samples represented old moisture damages that had dried out. Finding amoebae in such dry samples can be explained by the ability of the amoebae to exist as drought-resistant cysts in adverse conditions (Hausmann et al. 2003).

This study shows that amoebae are members of the microbial network occurring on moisture-damaged building materials. Still, the mere presence of a few amoebae in a sample of building material or in indoor air alone cannot confirm that the sample or the building is currently moisture-damaged. Amoebae are universally present in the outdoor environments and are likely to be transported indoors all the time, for example in the remains of natural water and soil in foodstuffs, shoe soles, and on the paws of pet animals. Amoebal cysts are often small enough to be airborne and can gain access to the indoor environment via the air. Thus, low numbers of amoebae may well be found also in healthy buildings. Nonetheless, high numbers of amoebae in an indoor environment would indicate that the organism has proliferated, and a high water content is required before that will happen. Therefore, an abundance of amoebae could indicate either a past or current high water content in the sample.

Similarly, this is also the case with fungal and bacterial species that are considered to be indicators of moisture damage in building materials (STM 2003). It is the combination of the presence of indicative species and increased microbial concentrations that together point to the indoor source of microbial growth. Amoebae could be placed among these indicators, so that their occurrence in conjunction with the other factors would strengthen the conclusion that a building has suffered a moisture damage. However, it needs to be borne in mind that in the current study amoebae were analyzed mainly from buildings with moisture-damage. Their occurrence and abundance in healthy buildings has not been studied, and thus there is no information on the "normal" amoebal levels. Thus, in order to link the abundance of amoebae to the possibility of moisture damage, more accurate methods for quantification of amoebae should be developed. Molecular methods are likely to make this possible in the future.

6.2 Amoebal survival in moisture-damaged buildings

The current study shows that acanthamoebae, the most ubiquitous free-living amoeba, are able to survive on many building materials used today when the materials become wet. Acanthamoebae are known to be highly adaptive and capable of flourishing in different environments (De Jonckheere 1991), which was confirmed in this study. Although the addition of supplementary nutrition on the material samples increased the percentage of amoebal survival, the amoebae survived on mineral insulation, gypsum board and old pine without additional sustenance. This finding suggests that amoebae are able to extract nutrients directly from these materials. Only the compounds released from fresh pine seemed to totally prevent amoebal survival, and although concrete and linoleum did not

support amoebal survival over the long term, amoebae were detected on these materials for as long as 14 days. Furthermore, even though acanthamoebae are perhaps the most universal free-living amoebae, it is likely that other amoebae would also be able to survive and even grow on moisture-damaged building materials. It is possible that other species would be able to utilize different niches in the building and thus the possibilities of amoebal survival would be higher. In practice this could mean that moisture content, not the choice of building materials, would be the factor limiting the occurrence of amoebae in moisture-damaged buildings.

In addition to moisture-damaged building materials, there are other places in the building where amoebae could also survive. Amoebae have been found in bathrooms and other sanitary areas in wash basin drains and water taps and in dust (Seal et al. 1992), and on the wall and floor tiles (Rohr et al. 1998). Amoebae have also been detected in aquariums (De Jonckheere 1979a), humidifiers (van Assendelft et al. 1979), in the soil of potted plants (Dunnebacke et al. 2004), and even in terrariums in the feces of pet lizards (Hassl and Benyr 2003). Other possible locations for amoebae could be basically any areas that at least occasionally become wet, such as the condensation plates used in fridges and freezers, or the floor just inside of the front door which can be made wet from water or snow on shoes.

6.3 The effects of amoebae on microbial exposure in moisture-damaged buildings

The current study shows that amoebae can interact with other microbes present in moisture-damaged buildings and this interaction may have effects on both the numbers and biological activity of these microbes. In this study, when *Acanthamoeba polyphaga* was grown together with *Streptomyces californicus*, *Pseudomonas fluorescens* and *Bacillus cereus*, the bacterial numbers increased considerably in co-culture compared to the bacteria grown alone. In contrast, effects on the fungi *Penicillium spinulosum*, *Stachybotrys chartarum* and *Aspergillus versicolor* were much less drastic and only slightly higher fungal counts were found in the presence of amoebae.

When assessing the changes in biological activity, it was found that co-culturing with amoebae increased the cytotoxicity and proinflammatory potential of the bacterium *Streptomyces californicus* and the fungus *Penicillium spinulosum*. For *S. californicus*, the effect was seen immediately after the onset of the interaction

whereas for *P. spinulosum* the effect built up slowly, peaking at the end of the experiment at 28 days. Both of these organisms can produce an impressive array of metabolic products, some of which are toxins (Anderson and Wellington 2001; Frisvad and Filtenborg 1983). To our knowledge, this is the first time when amoebae have been shown to increase the proinflammatory potential of a fungus and a non-pathogenic bacterium.

In practice, these findings indicate that amoebae might alter the exposure in moisture-damage buildings both quantitatively and qualitatively. The presence of amoebae in the moisture-damaged site would benefit the bacteria that are able to infect and replicate within amoebal cells, resulting in an increase of their relative numbers compared to non-intracellular bacteria. This could lead to a predominance of bacteria that might also be able to infect humans. Furthermore, intracellular growth within amoebae acts as a "training ground", making bacteria and possibly also fungi more efficient at infecting human and animal cells and causing disease in animal models (Cirillo et al. 1994; 1997; 1999; Molmeret et al. 2005; Steenbergen et al. 2003).

On the other hand, it is possible that the presence of amoebae in a moisture-damaged building might increase the toxicity or inflammatory properties of the bacteria and fungi even though microbial numbers are not necessarily affected, as shown in this study. Intracellular mechanisms are not the only option; also the amoebal selective grazing and production of metabolites can stimulate bacterial and possibly fungal growth (Jjemba 2001; Rønn et al. 2002) and also many bacteria and fungi are able to defend themselves by producing metabolites that are harmful to amoebae (Andersson et al. 1997). It is not clear which mechanisms are involved in the increase in inflammatory potency after co-culture with amoebae shown in this study. Future studies are needed to elucidate whether intracellular replication takes place, and which metabolic products are released during this interaction.

Amoebae could also serve as vectors for moving bacteria and fungal spores from one location to another, either intracellularly or ectocellularly on their surface. This dispersion could take place within the building, between individual moist sites, but also from outdoors to indoors. As many as 25 % of environmental amoebae are estimated to carry endosymbiotic bacteria (Fritsche et al. 1993), some of which may not survive without a host organism (Amann et al. 1997; Birtles et al. 1997; 2000). These microbes could be introduced into buildings inside amoebae. Many of these intracellular bacteria are potential respiratory pathogens (Corsaro and Venditti 2004) and thus they may have significance in the development of health effects in humans living in moisture-damaged buildings.

6.4 Methodological considerations

6.4.1 Culturing methods of fungi and bacteria (I, III, IV)

In the microbial analysis of samples from moisture-damaged buildings, two culturing methods were used for fungi and bacteria (I). The samples analyzed in the National Public Health Institute were solely cultivated with the dilution plating method, whereas in the Finnish Institute of Occupational Health, the samples were mainly cultured with a direct plating method. Both methods reflect the concentration of viable spores or bacterial cells on the building materials. The dilution plating method estimates the number of colony forming units per weight of sample, while direct plating is only semiquantitative with 4-step grading of abundance. Furthermore, the fungal genera detected with these two methods are slightly different, with perhaps more species being detected with the direct culture method (Verhoeff et al. 1994). Despite these differences, both methods are reported to provide similar results when evaluating the samples for their moisture damage (Reiman et al. 1999). To overcome the differences in the type of the method outcomes in this study, the results of both methods for each fungal genera were condensed to either a 3-step classification, namely “no growth”, “some growth” and “abundant growth” or to dichotomous presence/absence data. Even with this inevitable loss of data, interesting correlations were found between both the abundance and the occurrence of amoebae and certain fungal genera. In the other two studies involving culturing of fungi and bacteria (III, IV), no such dilemma existed because only dilution plating was used to enumerate the concentrations of known strains of microbes.

The quantification and identification of the fungal and bacterial flora is dependent on the culture media and conditions used (Hyvärinen 2002). In this study, the samples analyzed in the National Public Health Institute were cultured on MEA and DG18 media for fungi and on TYG medium for bacteria (Studies I, III, IV). In addition, the samples analyzed in the Finnish Institute of Occupational Health were also cultured on Hagem media for fungi (I). In the comparison of the fungal media, MEA and Hagem are suitable for fungi with higher moisture requirements, whereas DG18 is suited for more xerophilic strains. Together these media are able to detect the majority of indoor fungi relevant for detecting possible moisture damage in the building (Samson et al. 1994), although fast-growing genera like *Penicillium* may overwhelm slower-growing genera such as *Stachybotrys* on MEA (Andersen and Nissen 2000).

6.4.2 Detection of amoebae on building material samples (I, II)

Amoebae were detected from building material samples with the modified NNA-method, i.e. placing a piece of the sample on non-nutritive agar streaked with heat-killed *E. coli* (Newsome et al. 1998). The bacterium was heat-killed to prevent outgrowth on the plate. In this method, amoebae migrate freely from the sample material onto the agar to feed on *E. coli* and they can be microscopically identified and enumerated on the plate. However, the number of amoebae counted on the plate does not exactly equal their number on the sample, because they are likely to reproduce during the incubation (3 days in this work). On the other hand, according to our pilot tests with old wood pieces, the number of amoebae on the plate after incubation is proportional – approximately one log number less – to the number of *Acanthamoebae* inoculated onto the sample (data not shown). Other genera might emerge from the samples at a different time scale and replicate with different cycles, thus possibly making the proportion different. The properties of the sample material might also affect the speed and the proportion of the amoebae migrating on the plate. To allow for such disparity, the NNA-method was only used either semiquantitatively (I) or just to detect the presence of amoebae on the sample (II) in this thesis work. Using this method for acquiring reliable numerical data on amoebal concentrations on building material samples would require it to be validated for a wide array of different building materials, amoebal species, numbers of inoculated amoebae, lengths of incubation, and incubation temperatures. Even if all these could be controlled, it might prove impossible to exactly enumerate the amoebae on the plate especially at the higher numbers, as amoebae tend to cluster on the *E. coli* lines. It will probably be better to adopt some other methodology for amoebal quantification, such as qPCR, and apply the NNA-method only for investigating the occurrence of different amoebal genera on the samples.

Furthermore, it was not possible to distinguish with this method whether the amoebae had merely survived on the material as a cyst form or had they actively replicated in the trophozoite form. This might be relevant in evaluating their role in the exposure in moisture-damaged buildings. One could postulate that amoebae in the trophozoite form may interact with the other microbes present and alter their properties more than dormant cysts.

6.4.3 Immunotoxicological analyses (IV)

Selection of cell line and proinflammatory mediators

Immunotoxicological analyses were performed in this study by exposing murine RAW264.7 macrophages to graded doses of fungi, bacteria and amoebae and measuring the production of IL-6, TNFα and NO after the exposure. Macrophages are the first-in-line defense cells against inhaled particulate matter, and are thus the most relevant cells with which to evaluate the effects of the respirable indoor microbes. The RAW264.7 cell line and the abovementioned proinflammatory mediators were selected based on earlier experiments performed in our department which have shown these to be most sensitive in describing the biological activity of indoor microbes (Huttunen et al. 2000; 2001; 2003; Penttinen et al. 2005a).

Selection of the doses and treatment of the samples

The doses of fungi and bacteria administered to macrophages were set to 3×10^4, 10^5 and 3×10^5 and expressed as the equivalent number of spores or bacterial cells. These doses were slightly lower than used earlier in similar experiments (Huttunen et al. 2003) due to the limited availability of the spores in the incubated samples. However, some differences were seen already at the middle dose between the amoeba-cocultivated and separately cultivated fungi or bacteria. No overloading should be expected with such doses of spores or cells (Huttunen 2003; Markkanen 2008).

In this experiment, amoebae and fungi and bacteria were co-cultivated for up to four weeks in an aqueous suspension. This led to two significant differences to earlier experiments conducted with indoor microbes in our department. First, the suspension water had to be replaced with HBSS to avoid the adverse effect of water on the macrophages. During this stage, some, possibly bioactive, metabolites of the microbes might have been lost. Secondly, there also were fungal (and streptomycete) hyphae present in the doses given to macrophages, because it was impossible to extract only the spores from the suspensions. The biomass of the hyphae was not estimated nor taken into account when adjusting the dosage. This could have had an effect on the responses measured from the RAW264.7 macrophages. When calculating the spore counts under the microscope, the amounts of fungal hyphal growth seemed similar in both separately and amoeba-cocultivated samples, although no values were calculated for the hyphae.

Due to these differences, it is difficult to compare the current results with those of earlier experiments. Even though the present measurements of concentrations of cytokines after exposure to fungi are in line with earlier studies, it is possible that the washing of the samples and the additional particulate load from hyphae might have cancelled each others' effects. For bacteria and especially for *Pseudomonas*, the concentrations of cytokines and NO were much lower in this experiment than previously, and this might be attributable to the possible loss of active components during the change of suspensions. Nonetheless, the main finding, i.e. the demonstrations that the cocultivation with amoebae could increase the activity of *Streptomyces* and *Penicillium*, is not compromised because the responses of the macrophages to similarly treated separately grown and amoeba-cocultivated fungi and bacteria are comparable.

6.5 Implications for future practice and research

This study has provoked several further questions about the importance of amoebae in moisture-damaged buildings. It would be important to find out whether the abundance of amoebae is related to the severity of moisture damage, which amoebal genera can be detected in buildings and whether there is any difference in their relative occurrence and growth requirements. In order to clarify the possible link to health effects, it would be necessary to explore in detail the mechanisms involved in the increase in the effects attributable to amoebal co-culture on bacterial and fungal growth and viability, and also to determine which metabolic products are released during this interaction, especially which are responsible for the increase in the proinflammatory potential. It will be also crucial to test epidemiologically whether the occurrence of amoebae in moisture-damaged buildings can be linked with any of the health effects and symptoms experienced by the occupants of moisture-damaged buildings.

In summary, there are many potential ways that amoebae may modulate the exposure situation within moisture-damaged buildings. The occurrence of amoebae should be taken into account when assessing the exposure of occupants in moisture-damaged buildings, but also when studying the mechanisms behind the health effects associated with exposure in these buildings.

7 CONCLUSIONS

1. Amoebae were detected in approximately 22 % of the building material samples taken from moisture-damaged buildings, showing that they are members of the microbial network occurring in these buildings. Amoebae occurred most often together with the kinds of fungi and bacteria that are considered indicators of moisture damage in buildings, such as *Streptomycetes* and the fungi *Aspergillus versicolor*, and *Trichoderma*. Furthermore, amoebae were detected almost exclusively on samples judged to be water-damaged. Thus, amoebae could be suggested to be one of the indicator microorganisms of moisture damage.

2. *Acanthamoeba polyphaga*, representing the free-living amoebae, were able to survive at least temporarily on most modern building materials. Thus, it is the moisture content rather than the choice of building material that limits the survival of amoebae in moisture-damaged buildings.

3. Co-culturing with *Acanthamoeba polyphaga* increased the growth of selected bacteria and to some extent, also of the fungi, commonly found in moisture-damaged buildings. Therefore, amoebae may have an effect on the quantity of microbial biomass on a material and hence, potentially on the exposure in moisture-damaged buildings.

4. The interaction between *Acanthamoeba polyphaga* and the bacterium *Streptomyces californicus* and the fungus *Penicillium spinulosum* resulted in an elevated cytotoxicity and synergistic increase in proinflammatory potential of these microbes. Consequently, amoebae may have effect on the biological activity of the microbes present in moisture-damaged buildings and thus also indirectly on the health effects experienced by the occupants.

8 ACKNOWLEDGEMENTS

This study was carried out in Department of Environmental Health, National Institute for Health and Welfare (former National Public Health Institute), in 2001-2009. During this time, the Department has been led by three Directors; Professors Jouko Tuomisto, Terttu Vartiainen, and Juha Pekkanen. My sincere thanks go to all three of them for providing the facilities for this study.

Main part of this study was funded by the Academy of Finland, but I was also lucky enough to receive financial support from Finnish Cultural Foundation of Northern Savo, Juho Vainio Foundation, The Finnish Work Environment Fund, Kuopio University Foundation, Kone Foundation, and National Public Health Institute. I wish to express my gratitude to the funders for making this study possible.

My principal supervisor, Research Professor Aino Nevalainen, deserves my deepest and warmest thanks for guiding me to the world of science and in it. I am grateful to her always offering guidance, support and encouragement, and for giving the time and space to do things in my way - in an optimal ratio! She is unsurpassed in her ability to see the big picture, but she also has the uncanny skill to spot the odd word out in minimal time in any text brought to her. No matter how good I thought the text was, she can always make it better! I am also extremely grateful to my other supervisors, Professor Maija-Riitta Hirvonen and Adjunct Professor Markku Seuri, for their valuable insights and advice. This study would not have been possible without their considerable input.

I sincerely thank the official reviewers of this thesis, Professor Emeritus Stuart Bamforth from University of Tulane, and Professor Rafal Górny from Institute of Occupational Medicine and Environmental Health, for their valuable comments and constructive criticism. Thanks also go to Ewen MacDonald for reviewing this thesis and the original articles for English.

I wish to thank all of the co-authors for their collaboration in this study. My special thanks are due to co-author and dear colleague Jaana Kusnetsov, who took me into her motherly care right in the beginning of this work. She has supported me in every way possible in all matters of science, work, children, and life in general! A big huge thank you to you! Thanks are also due to Asko Vepsäläinen and Pekka Tiittanen for much appreciated help with the statistics.

I owe my warmest thanks to laboratory personnel helping out in this study: Marjo Tiittanen, Heli Martikainen, Pirkko Karakorpi, Ulla Kukkonen, Arja Rönkkö and Sirpa Lappalainen. Without you I would still be in the lab, pipetting and plating,

again and again. Thanks are also due to Kirsi Korhonen, our ever so efficient secretary, who always helps with a smile and a kind word. Thank you all for your professional expertise and helpful attitude! The “mold group” and whole former YMB laboratory is acknowledged for being such pleasant colleagues - thank you.

I have been lucky the share an office with three wonderful women: Jaana, Helena, and Hanna. Thank you all for those sometimes lengthy discussions, that occasionally even were about work! It has been fun to share the room with each of you. And I promise to keep singing the favourite song of the current room-mate Hanna with great gusto: *Yes it’s gonna be cold, lonely summer*…

Thanks are due to the fantastic group of people who have enlightened my work days with their quirky sense of humour, administered over a cup of coffee: Marjo, Outi, Katja, Maria, Martin, Helena, Mari, to name just a few. I must have had many extra years to my life for having laughed so hard at your oddball comments. I’ll never forget the one about not having all the jam in the doughnut! Thank you and keep the coffee hot!

My dear friends, Niina, Hani and Ciitu, have borne with me for longer than I remember. Even though the contact has sometimes been patchy, every time we see it’s like we met yesterday. Thank you for being there and accepting me as I am, even though you know all my faults!

I want to thank my college biology teacher, James Mendelssohn, for guiding me to scientific thought by never giving any direct answers, and questioning everything I thought certain. I really enjoyed the classes - learning by frustration seemed to work. Although, I still do not understand how the squirrel knew which part of the conker to bite!

I owe many thanks to my uncles Ilkka and Jouko, to my mother Armi, and to my late grandmother Anja. They have always believed in me and given love and support throughout my life. Thank you.

The last and the most important thanks go to my family, my dear husband Pasi and our lovely daughter Elli. You are the treasure of my life. There are no words to express the depth of my gratitude of having you in my life so I just say thank you for everything.

9 REFERENCES

Abbas AK, Lichtman AH, Pober JS. 2000. Cellular and molecular immunology. 4th. ed. Saunders Company: Philadelphia, USA.

Abd H, Johansson T, Golovliov I, Sandström G, Forsman M. 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Applied and Environmental Microbiology,* **69**:600-606.

Abd H, Weintraub A, Sandström G. 2005. Intracellular survival and replication of *Vibrio cholerae* O139 in aquatic free-living amoebae. *Environmental Microbiology,* **7**:1003-1008.

Abd H, Saeed A, Weintraub A, Nair GB, Sandström G. 2007. *Vibrio cholerae* O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. *FEMS Microbiology Ecology,* **60**:33-39.

Adékambi T, Reynaud-Gaubert M, Greub G, Gevaudan MJ, La Scola B, Raoult D, Drancourt M. 2004. Amoebal coculture of "*Mycobacterium massiliense*" sp. nov. from the sputum of a patient with hemoptoic pneumonia. *Journal of Clinical Microbiology,* **42**:5493-5501.

Adékambi T, Ben Salah S, Khlif M, Raoult D, Drancourt M. 2006. Survival of environmental mycobacteria in *Acanthamoeba polyphaga*. *Applied and Environmental Microbiology,* **72**:5974-5981.

Adeleke A, Pruckler J, Benson R, Rowbotham T, Halablab M, Fields B. 1996. Legionella-like amebal pathogens - phylogenetic status and possible role in respiratory disease. *Emerging Infectious Diseases,* **2**:225-230.

Adeleke AA, Fields BS, Benson RF, Daneshvar MI, Pruckler JM, Ratcliff RM, Harrison TG, Weyant RS, Birtles RJ, Raoult D, Halablab MA. 2001. *Legionella drozanskii* sp. nov., *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov.: three unusual new *Legionella* species. *International Journal of Systematic and Evolutionary Microbiology,* **51**:1151-1160.

Adl SM, Simpson AGB, Farmer MA, Andersen RA, Anderson OR, Barta JR, Bowser SS, Brugerolle G, Fensome RA, Fredericq S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn DH, Mann DG, McCourt RM, Mendoza L, Moestrup O, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor MFJR. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology,* **52**:399-451.

Aimard L, Brasseur P, Favennec L, Perrine D, Watt L, Brasseur G. 1998. Amebic keratitis due to a mixed infection with *Acanthamoeba* and *Hartmanella* species. *Clinical Infectious Diseases,* **26**:187-188.

Aitken D, Hay J, Kinnear FB, Kirkness CM, Lee WR, Seal DV. 1996. Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmannella* infection. *Ophthalmology,* **103**:485-494.

Aksozek A, McClellan K, Howard K, Niederkorn JY, Alizadeh H. 2002. Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. *Journal of Parasitology,* **88**:621-623.

Alexandrakis G, Miller D, Huang AJ. 1998. Amebic keratitis due to *Vahlkampfia* infection following corneal trauma. *Archives of Ophthalmology,* **116**:950-951.

Amann R, Springer N, Schonhuber W, Ludwig W, Schmid EN, Muller KD, Michel R. 1997. Obligate intracellular bacterial parasites of *Acanthamoebae* related to *Chlamydia* spp. *Applied and Environmental Microbiology,* **63**:115-121.

Anaya-Velazquez F, Padilla-Vaca F. 1992. Effect of intestinal bacteria on the virulence of *Entamoeba histolytica*. *Archives of Medical Research,* **23**:183-185.

Andersen B, Nissen AT. 2000. Evaluation of media for detection of *Stachybotrys* and *Chaetomium* species associated with water-damaged buildings. *International Biodeterioration & Biodegradation,* **46**:111-116.

Anderson AS, Wellington EMH. 2001. The taxonomy of *Streptomyces* and related genera. *International Journal of Systematic and Evolutionary Microbiology,* **51**:797-814.

Anderson IJ, Watkins RF, Samuelson J, Spencer DF, Majoros WH, Gray MW, Loftus BJ. 2005. Gene discovery in the *Acanthamoeba castellanii* genome. *Protist,* **156**:203-214.

Anderson OR, Rogerson A, Hannah F. 1997. Three new limax amoebae isolated from marine surface sediments: *Vahlkampfia caledonica* n. sp., *Saccamoeba marina* n. sp., and *Hartmannella vacuolata* n. sp. *Journal of Eukaryotic Microbiology,* **44**:33-42.

Anderson OR. 1998. Densities and diversity of gymnamoebae in relation to some inshore aquatic habitats at Bermuda. *Journal of Eukaryotic Microbiology,* **45**:151-155.

Andersson MA, Nikulin M, Köljalg U, Andersson MC, Rainey F, Reijula K, Hintikka E-L, Salkinoja-Salonen M. 1997. Bacteria, molds, and toxins in water-damaged building materials. *Applied and Environmental Microbiology,* **63**:387-393.

Arias Fernandez MC, Paniagua Crespo E, Marti Mallen M, Penas Ares MP, Casro Casas ML. 1989. Marine amoebae from waters of northwest Spain, with comments on a potentially pathogenic euryhaline species. *Journal of Protozoology,* **36**:239-241.

Armstrong E, Rogerson A, Leftley JW. 2000. The abundance of heterotrophic protists associated with intertidal seaweeds. *Estuarine Coastal and Shelf Science*, **50**:415-424.

Axelsson-Olsson D, Waldenstrom J, Broman T, Olsen B, Holmberg M. 2005. Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. *Applied and Environmental Microbiology*, **71**:987-992.

Bakke JV, Norbäck D, Wieslander G, Hollund BE, Moen BE. 2007. Pet keeping and dampness in the dwelling: associations with airway infections, symptoms, and physiological signs from the ocular and nasal mucosa. *Indoor Air*, **17**:60-69.

Bamforth SS, Curds CR, Finlay BJ. 1987. Protozoa of two Kenya Lakes. *Transactions of the American Microscopical Society*, **106**:354-358.

Bamforth SS. 2004. Water film fauna of microbiotic crusts of a warm desert. *Journal of Arid Environments*, **56**:413-423.

Bamforth SS, Wall DH, Virginia RA. 2005. Distribution and diversity of soil protozoa in the McMurdo Dry Valleys of Antarctica. *Polar Biology*, **28**:756-762.

Barbeau J, Buhler T. 2001. Biofilms augment the number of free-living amoebae in dental unit waterlines. *Research in Microbiology*, **152**:753-760.

Barker J, Brown MR. 1995. Speculations on the influence of infecting phenotype on virulence and antibiotic susceptibility of *Legionella pneumophila*. *Journal of Antimicrobial Chemotherapy*, **36**:7-21.

Barker J, Humphrey TJ, Brown MW. 1999. Survival of *Escherichia coli* O157 in a soil protozoan: implications for disease. *FEMS Microbiology Letters*, **173**:291-295.

Barnes PJ, Chung KF, Page CP. 1998. Inflammatory mediators of asthma: an update. *Pharmacological Reviews*, **50**:515-596.

Baumgartner M, Yapi A, Gröbner-Ferreira R, Stetter KO. 2003. Cultivation and properties of *Echinamoeba thermarum* n. sp., an extremely thermophilic amoeba thriving in hot springs. *Extremophiles*, **7**:267-274.

Befinger M, Myjak P, Pietkiewicz H. 1986. Occurrence of amphizoic amoebae in Lake Zarnowieckie. *Bulletin of the Institute of Maritime and Tropical Medicine in Gdynia*, **37**:275-286.

Behets J, Declerck P, Delaedt Y, Verelst L, Ollevier F. 2007. Survey for the presence of specific free-living amoebae in cooling waters from Belgian power plants. *Parasitology Research*, **100**:1249-1256.

Belanger K, Beckett W, Triche E, Bracken MB, Holford T, Ren P, McSharry JE, Gold DR, Platts-Mills TAE, Leaderer BP. 2003. Symptoms of wheeze and persistent cough in the first year of life: Associations with indoor allergens, air contaminants, and maternal history of asthma. *American Journal of Epidemiology*, **158**:195-202.

Belmadani A, Steyn PS, Tramu G, Betbeder AM, Baudrimont I, Creppy EE. 1999. Selective toxicity of ochratoxin A in primary cultures from different brain regions. *Archives of Toxicology,* **73**:108-114.

Bermingham ML, Mulcahy MF. 2007. *Neoparamoeba* sp. and other protozoans on the gills of Atlantic salmon *Salmo salar* smolts in seawater. *Diseases of Aquatic Organisms,* **76**:231-240.

Birtles RJ, Rowbotham TJ, Storey C, Marrie TJ, Raoult D. 1997. Chlamydia-like obligate parasite of free-living amoebae. *Lancet,* **349**:925-926.

Birtles RJ, Rowbotham TJ, Michel R, Pitcher DG, La Scola B, Alexiou-Daniel S, Raoult D. 2000. '*Candidatus* Odyssella thessalonicensis' gen. nov., sp. nov., an obligate intracellular parasite of *Acanthamoeba* species. *International Journal of Systematic and Evolutionary Microbiology,* **50 Pt 1**:63-72.

Booton GC, Schuster FL, Carmichael JR, Fuerst PA, Byers TJ. 2003. *Balamuthia mandrillaris*: Identification of clinical and environmental isolates using genus-specific PCR. *Journal of Eukaryotic Microbiology,* **50**:508-509.

Bornehag CG, Blomquist G, Gyntelberg F, Jarvholm B, Malmberg P, Nordvall L, Nielsen A, Pershagen G, Sundell J. 2001. Dampness in buildings and health - Nordic interdisciplinary review of the scientific evidence on associations between exposure to "dampness" in buildings and health effects (NORDDAMP). *Indoor Air,* **11**:72-86.

Bornehag CG, Sundell J, Bonini S, Custovic A, Malmberg P, Skerfving S, Sigsgaard T, Verhoeff A. 2004. Dampness in buildings as a risk factor for health effects, EUROEXPO: a multidisciplinary review of the literature (1998-2000) on dampness and mite exposure in buildings and health effects. *Indoor Air,* **14**:243-257.

Bornehag CG, Sundell J, Hagerhed-Engman L, Sigsgaard T, Janson S, Aberg N. 2005. 'Dampness' at home and its association with airway, nose, and skin symptoms among 10,851 preschool children in Sweden: a cross-sectional study. *Indoor Air,* **15**:S48-55.

Bracha R, Mirelman D. 1984. Virulence of *Entamoeba histolytica* trophozoites. Effects of bacteria, microaerobic conditions, and metronidazole. *Journal of Experimental Medicine,* **160**:353-368.

Brasel TL, Martin JM, Carriker CG, Wilson SC, Straus DC. 2005. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins in the indoor environment. *Applied and Environmental Microbiology,* **71**:7376-7388.

Brown S, De Jonckheere JF. 2004. Isolation of a new vahlkampfiid amoeba from soil: *Paravahlkampfia lenta* n. sp. *European Journal of Protistology,* **40**:289-294.

Brown TJ, Cursons RT, Keys EA. 1982. Amoebae from Antarctic soil and water. *Applied and Environmental Microbiology,* **44**:491-493.

Burr ML, Matthews IP, Arthur RA, Watson HL, Gregory CJ, Dunstan FDJ, Palmer SR. 2007. Effects on patients with asthma of eradicating visible indoor mould: a randomised controlled trial. *Thorax,* **62**:766-771.

Byers TJ, Akins RA, Maynard BJ, Lefken RA, Martin SM. 1980. Rapid growth of *Acanthamoeba* in defined media; induction of encystment by glucose-acetate starvation. *Journal of Protozoology,* **27**:216-219.

Carter RF. 1970. Description of a *Naegleria* sp. isolated from two cases of primary amoebic meningo-encephalitis, and of the experimental pathological changes induced by it. *Journal of Pathology,* **100**:217-244.

CDC. 2007. http://www.cdc.gov/ncidod/dpd/parasites/acanthamoeba/index.htm. Centers for Disease Control and Prevention; p. Official www-page for Acanthamoeba infection.

Cenci E, Mencacci A, Casagrande A, Mosci P, Bistoni F, Romani L. 2001. Impaired antifungal effector activity but not inflammatory cell recruitment in interleukin-6-deficient mice with invasive pulmonary aspergillosis. *Journal of Infectious Diseases,* **184**:610-617.

Cengiz AM, Harmis N, Stapleton F. 2000. Co-incubation of *Acanthamoeba castellanii* with strains of *Pseudomonas aeruginosa* alters the survival of amoeba. *Clinical & Experimental Ophthalmology,* **28**:191-193.

Chagla AH, Griffiths AJ. 1974. Growth and encystation of *Acanthamoeba castellanii*. *Journal of General Microbiology,* **85**:139-145.

Charpin-Kadouch C, Maurel G, Felipo R, Queralt J, Ramadour M, Dumon H, Garans M, Botta A, Charpin D. 2006. Mycotoxin identification in moldy dwellings. *Journal of Applied Toxicology,* **26**:475-479.

Chávez-Munguía B, Omana-Molina M, González-Lázaro M, González-Robles A, Bonilla P, Martínez-Palomo A. 2005. Ultrastructural study of encystation and excystation in *Acanthamoeba castellanii*. *Journal of Eukaryotic Microbiology,* **52**:153-158.

Chisholm RL, Gaudet P, Just EM, Pilcher KE, Fey P, Merchant SN, Kibbe WA. 2006. dictyBase, the model organism database for *Dictyostelium discoideum*. *Nucleic Acids Research,* **34**:D423-427.

Cho SH, Reponen T, LeMasters G, Levin L, Huang J, Meklin T, Ryan P, Villareal M, Bernstein D. 2006. Mold damage in homes and wheezing in infants. *Annals of Allergy Asthma & Immunology,* **97**:539-545.

Cirillo JD, Falkow S, Tompkins LS. 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity,* **62**:3254-3261.

Cirillo JD, Falkow S, Tompkins LS, Bermudez LE. 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity,* **65**:3759-3767.

Cirillo JD. 1999. Exploring a novel perspective on pathogenic relationships. *Trends in Microbiology*, **7**:96-98.

Cirillo JD, Cirillo SL, Yan L, Bermudez LE, Falkow S, Tompkins LS. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infection and Immunity*, **67**:4427-4434.

Collingro A, Walochnik J, Baranyi C, Michel R, Wagner M, Horn M, Aspöck H. 2004. Chlamydial endocytobionts of free-living amoebae differentially affect the growth rate of their hosts. *European Journal of Protistology*, **40**:57-60.

Collingro A, Toenshoff ER, Taylor MW, Fritsche TR, Wagner M, Horn M. 2005. '*Candidatus* Protochlamydia amoebophila', an endosymbiont of *Acanthamoeba* spp. *International Journal of Systematic and Evolutionary Microbiology*, **55**:1863-1866.

Cordovilla P, Valdivia E, Gonzalez-Segura A, Galvez A, Martinez-Bueno M, Maqueda M. 1993. Antagonistic action of the bacterium *Bacillus licheniformis* M-4 toward the amoeba *Naegleria fowleri*. *Journal of Eukaryotic Microbiology*, **40**:323-328.

Corsaro D, Venditti D. 2004. Emerging chlamydial infections. *Critical Reviews in Microbiology*, **30**:75-106.

Crago BR, Nelson LA, Davis M, Arnold L, Thrasher JD. 2003. Psychological, neuropsychological, and electrocortical effects of mixed mold exposure. *Archives of Environmental Health*, **58**:452-463.

Dales RE, Burnett R, Zwanenburg H. 1991. Adverse health effects among adults exposed to home dampness and molds. *American Review of Respiratory Disease*, **143**:505-509.

Danes L, Cerva L. 1981. Survival of polioviruses and echoviruses in *Acanthamoeba castellanii* cultivated in vitro. *Journal of Hygiene, Epidemiology, Microbiology and Immunology*, **25**:169-174.

De Jonckheere JF. 1979a. Occurrence of *Naegleria* and *Acanthamoeba* in aquaria. *Applied and Environmental Microbiology*, **38**:590-593.

De Jonckheere JF. 1979b. Pathogenic free-living amoebae in swimming pools: survey in Belgium. *Annales de Microbiologie*, **130B**:205-212.

De Jonckheere JF, Michel R. 1988. Species identification and virulence of *Acanthamoeba* strains from human nasal mucosa. *Parasitology Research*, **74**:314-316.

De Jonckheere JF. 1991. Ecology of *Acanthamoeba*. *Reviews of Infectious Diseases*, **13**:S385-387.

De Jonckheere JF. 2002. A century of research on the amoeboflagellate genus *Naegleria*. *Acta Protozoologica*, **41**:309-342.

De Jonckheere JF, Brown S. 2005. Isolation of a Vahlkampfiid amoeba from a contact lens: *Tetramitus ovis* (Schmidt, 1913) n. comb. *European Journal of Protistology*, **41**:93-97.

De Jonckheere JF. 2007. Molecular identification of free-living amoebae of the Vahlkampfiidae and Acanthamoebidae isolated in Arizona (USA). *European Journal of Protistology*, **43**:9-15.

Declerck P, Behets J, Delaedt Y, Margineanu A, Lammertyn E, Ollevier F. 2005. Impact of non-*Legionella* bacteria on the uptake and intracellular replication of *Legionella pneumophila* in *Acanthamoeba castellanii* and *Naegleria lovaniensis*. *Microbial Ecology*, **50**:536-549.

Declerck P, Behets J, van Hoef V, Ollevier F. 2007. Detection of *Legionella* spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. *Water Research*, **41**:3159-3167.

Dey R, Bodennec J, Mameri MO, Pernin P. 2009. Free-living freshwater amoebae differ in their susceptibility to the pathogenic bacterium Legionella pneumophila. *FEMS Microbiology Letters*, **290**:10-17.

Dharmage S, Bailey M, Raven J, Abeyawickrama K, Cao D, Guest D, Rolland J, Forbes A, Thien F, Abramson M, Walters EH. 2002. Mouldy houses influence symptoms of asthma among atopic individuals. *Clinical and Experimental Allergy*, **32**:714-720.

Drancourt M, Adékambi T, Raoult D. 2007. Interactions between *Mycobacterium xenopi*, amoeba and human cells. *Journal of Hospital Infection*, **65**:138-142.

Drozanski WJ. 1991. *Sarcobium lyticum* Gen. Nov., Sp. Nov., an obligate intracellular bacterial parasite of small free-living amebas. *International Journal of Systematic Bacteriology*, **41**:82-87.

Dunnebacke TH, Schuster FL, Yagi S, Booton GC. 2003. Isolation of *Balamuthia* amebas from the environment. *Journal of Eukaryotic Microbiology*, **50**:S510-511.

Dunnebacke TH, Schuster FL, Yagi S, Booton GC. 2004. *Balamuthia mandrillaris* from soil samples. *Microbiology*, **150**:2837-2842.

Dyková I, Veverkova M, Fiala I, Machackova B, Peckova H. 2003a. *Nuclearia pattersoni* sp. n. (Filosea), a new species of amphizoic amoeba isolated from gills of roach (*Rutilus rutilus*), and its rickettsial endosymbiont. *Folia Parasitologica*, **50**:161-170.

Dyková I, Fiala I, Lom J, Lukes J. 2003b. *Perkinsiella amoebae*-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*. *European Journal of Protistology*, **39**:37-52.

Dyková I, Lom J. 2004. Advances in the knowledge of amphizoic amoebae infecting fish. *Folia Parasitologica*, **51**:81-97.

Ebbehøj NE, Meyer HW, Würtz H, Suadicani P, Valbjørn O, Sigsgaard T, Gyntelberg F. 2005. Molds in floor dust, building-related symptoms, and lung function among male and female schoolteachers. *Indoor Air,* **15**:S7-16.

Ekelund F, Rønn R. 1994. Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amebas and their ecology. *FEMS Microbiology Reviews,* **15**:321-353.

Engvall K, Norrby C, Norback D. 2002. Ocular, airway, and dermal symptoms related to building dampness and odors in dwellings. *Archives of Environmental Health,* **57**:304-310.

Essig A, Heinemann M, Simnacher U, Marre R. 1997. Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Applied and Environmental Microbiology,* **63**:1396-1399.

Ettinger MR, Webb SR, Harris SA, McIninch SP, Garman GC, Brown BL. 2003. Distribution of free-living amoebae in James River, Virginia, USA. *Parasitology Research,* **89**:6-15.

Fahrni JF, Bolivar I, Berney U, Nassonova E, Smirnov A, Pawlowski J. 2003. Phylogeny of lobose amoebae based on actin and small-subunit ribosomal RNA genes. *Molecular Biology and Evolution,* **20**:1881-1886.

Fischer A, Folkerts G, Geppetti P, Groneberg DA. 2002. Mediators of asthma: nitric oxide. *Pulmonary Pharmacology and Therapeutics,* **15**:73-81.

Flannigan B, Miller JD. 2001. Microbial Growth in Indoor Environments. In: Microorganisms in Home and Indoor Work Environments. (Ed Flannigan B, Samson RA, Miller JD). London: Taylor & Francis.

Flannigan B. 2001. Microorganisms in Indoor Air. In: Microorganisms in Home and Indoor Work Environments. (Ed Flannigan B, Samson RA, Miller JD). London: Taylor & Francis.

Fogh J, Holmgren NB, Ludovici PP. 1971. A review of cell culture contaminations. *In Vitro,* **7**:26-41.

Frisvad JC, Filtenborg O. 1983. Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. *Applied and Environmental Microbiology,* **46**:1301-1310.

Fritsche TR, Gautom RK, Seyedirashti S, Bergeron DL, Lindquist TD. 1993. Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from corneal and environmental specimens and contact lenses. *Journal of Clinical Microbiology,* **31**:1122-1126.

Fritsche TR, Sobek D, Gautom RK. 1998. Enhancement of in vitro cytopathogenicity by *Acanthamoeba* spp. following acquisition of bacterial endosymbionts. *FEMS Microbiology Letters,* **166**:231-236.

Fritsche TR, Horn M, Seyedirashti S, Gautom RK, Schleifer KH, Wagner M. 1999. In situ detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the order *Rickettsiales*. *Applied and Environmental Microbiology*, **65**:206-212.

Fritsche TR, Horn M, Wagner M, Herwig RP, Schleifer KH, Gautom RK. 2000. Phylogenetic diversity among geographically dispersed *Chlamydiales* endosymbionts recovered from clinical and environmental isolates of *Acanthamoeba* spp. *Applied and Environmental Microbiology*, **66**:2613-2619.

Gast RJ, Ledee DR, Fuerst PA, Byers TJ. 1996. Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. *Journal of Eukaryotic Microbiology*, **43**:498-504.

Gaze WH, Burroughs N, Gallagher MP, Wellington EM. 2003. Interactions between *Salmonella typhimurium* and *Acanthamoeba polyphaga*, and observation of a new mode of intracellular growth within contractile vacuoles. *Microbial Ecology*, **46**:358-369.

Gent JF, Ren P, Belanger K, Triche E, Bracken MB, Holford TR, Leaderer BP. 2002. Levels of household mold associated with respiratory symptoms in the first year of life in a cohort at risk for asthma. *Environmental Health Perspectives*, **110**:A781-A786.

Gilbert D, Mitchell EAD, Amblard C, Bourdier G, Francez AJ. 2003. Population dynamics and food preferences of the testate amoeba *Nebela tincta major-bohemica-collaris* complex (Protozoa) in a Sphagnum peatland. *Acta Protozoologica*, **42**:99-104.

Glushakova AM, Zheltikova TM, Chernov I. 2004. Groups and sources of yeasts in house dust. *Microbiology*, **73**:94-98.

Gordon WA, Cantor JB, Johanning E, Charatz HJ, Ashman TA, Breeze JL, Haddad L, Abramowitz S. 2004. Cognitive impairment associated with toxigenic fungal exposure: A replication and extension of previous findings. *Applied Neuropsychology*, **11**:65-74.

Górny RL, Dutkiewicz J, Krysínska-Traczyk E. 1999. Size distribution of bacterial and fungal bioaerosols in indoor air. *Annals of Agricultural and Environmental Medicine*, **6**:105-113.

Gottschalk C, Bauer J, Meyer K. 2008. Detection of satratoxin G and H in indoor air from a water-damaged building. *Mycopathologia*, **166**:103-107.

Goy G, Thomas V, Rimann K, Jaton K, Prod'hom G, Greub G. 2007. The Neff strain of *Acanthamoeba castellanii*, a tool for testing the virulence of *Mycobacterium kansasii*. *Research in Microbiology*, **158**:393-397.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Analytical Biochemistry*, **126**:131-138.

Greub G, La Scola B, Raoult D. 2003. *Parachlamydia acanthamoeba* is endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation temperature. *Annals of the New York Academy of Sciences,* **990**:628-634.

Greub G, La Scola B, Raoult D. 2004. Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture. *Emerging Infectious Diseases,* **10**:470-477.

Greub G, Raoult D. 2004. Microorganisms resistant to free-living amoebae. *Clinical Microbiology Reviews,* **17**:413-433.

Griffin JL. 1983. The pathogenic amoeboflagellate *Naegleria fowleri*: environmental isolations, competitors, ecologic interactions, and the flagellate-empty habitat hypothesis. *Journal of Protozoology,* **30**:403-409.

Hagnere C, Harf C. 1993. Symbiotic interactions between free-living ameba and harbored mercury-resistant bacteria. *European Journal of Protistology,* **29**:155-159.

Hall J, Voelz H. 1985. Bacterial endosymbionts of *Acanthamoeba* sp. *Journal of Parasitology,* **71**:89-95.

Hassl A, Benyr G. 2003. Hygienic evaluation of terraria inhabited by amphibians and reptiles: cryptosporidia, free-living amebas, salmonella. *Wiener Klinische Wochenschrift,* **115**:S68-71.

Hausmann K, Hulsmann N, Radek R. 2003. Protistology. 3rd. ed. E. Schweizerbart'sche Verlagsbuchhandlung: Berlin.

Haverinen U. 2002. Modeling Moisture Damage Observations and Their Association with Health Symptoms. In: Publications of the National Public Health Institute A10. Kuopio, Finland: National Public Health Institute; p. 106.

He GB, Yan N. 2005. Effect of wood on the curing behavior of commercial phenolic resin systems. *Journal of Applied Polymer Science,* **95**:185-192.

Heinz E, Kolarov I, Kästner C, Toenshoff ER, Wagner M, Horn M. 2007. An *Acanthamoeba* sp. containing two phylogenetically different bacterial endosymbionts. *Environmental Microbiology,* **9**:1604-1609.

Hewett MK, Robinson BS, Monis PT, Saint CP. 2003. Identification of a new *Acanthamoeba* 18S rRNA gene sequence type, corresponding to the species *Acanthamoeba jacobsi* Sawyer, Nerad and Visvesvara, 1992 (Lobosea : Acanthamoebidae). *Acta Protozoologica,* **42**:325-329.

Hirvonen MR, Nevalainen A, Makkonen N, Mönkkönen J, Savolainen K. 1997a. Induced production of nitric oxide, tumor necrosis factor, and interleukin-6 in RAW 264.7 macrophages by streptomycetes from indoor air of moldy houses. *Archives of Environmental Health,* **52**:426-432.

Hirvonen MR, Nevalainen A, Makkonen N, Mönkkönen J, Savolainen K. 1997b. Streptomyces spores from mouldy houses induce nitric oxide, TNF alpha and IL-6 secretion from RAW264.7 macrophage cell line without causing subsequent cell death. *Environmental Toxicology and Pharmacology,* **3**:57-63.

Hirvonen MR, Ruotsalainen M, Roponen M, Hyvärinen A, Husman T, Kosma VM, Komulainen H, Savolainen K, Nevalainen A. 1999. Nitric oxide and proinflammatory cytokines in nasal lavage fluid associated with symptoms and exposure to moldy building microbes. *American Journal of Respiratory and Critical Care Medicine,* **160**:1943-1946.

Hoffmann R, Michel R, Schmid EN, Muller KD. 1998. Natural infection with microsporidian organisms (KW19) in *Vannella* spp. (Gymnamoebia) isolated from a domestic tap-water supply. *Parasitology Research,* **84**:164-166.

Hoffmann R, Michel R. 2001. Distribution of free-living amoebae (FLA) during preparation and supply of drinking water. *International Journal of Hygiene and Environmental Health,* **203**:215-219.

Holden EP, Winkler HH, Wood DO, Leinbach ED. 1984. Intracellular growth of *Legionella pneumophila* within *Acanthamoeba castellanii* Neff. *Infection and Immunity,* **45**:18-24.

Holgate ST, Davies DE, Puddicombe S, Richter A, Lackie P, Lordan J, Howarth P. 2003. Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. *European Respiratory Journal. Supplement,* **44**:24s-29s.

Hookey JV, Saunders NA, Fry NK, Birtles RJ, Harrison TG. 1996. Phylogeny of *Legionellaceae* based on small-subunit ribosomal DNA sequences and proposal of *Legionella lytica* comb nov for *Legionella*-Like amoebal pathogens. *International Journal of Systematic Bacteriology,* **46**:526-531.

Horn M, Fritsche TR, Gautom RK, Schleifer KH, Wagner M. 1999. Novel bacterial endosymbionts of *Acanthamoeba* spp. related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus. Environmental Microbiology,* **1**:357-367.

Horn M, Wagner M, Müller KD, Schmid EN, Fritsche TR, Schleifer KH, Michel R. 2000. *Neochlamydia hartmannellae* gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis. Microbiology,* **146**:1231-1239.

Horn M, Harzenetter MD, Linner T, Schmid EN, Müller KD, Michel R, Wagner M. 2001. Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum as intracellular bacteria of acanthamoebae: proposal of *'Candidatus* Amoebophilus asiaticus'. *Environmental Microbiology,* **3**:440-449.

Horn M, Fritsche TR, Linner T, Gautom RK, Harzenetter MD, Wagner M. 2002. Obligate bacterial endosymbionts of *Acanthamoeba* spp. related to the β-Proteobacteria: proposal of *'Candidatus* Procabacter acanthamoebae' gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **52**:599-605.

Horner WE, Helbling A, Salvaggio JE, Lehrer SB. 1995. Fungal allergens. *Clinical Microbiology Reviews,* **8**:161-179.

Hundt MJ, Ruffolo CG. 2005. Interaction of *Pasteurella multocida* with free-living amoebae. *Applied and Environmental Microbiology*, **71**:5458-5464.

Huttunen K, Ruotsalainen M, Iivanainen E, Torkko P, Katila ML, Hirvonen MR. 2000. Inflammatory responses in RAW264.7 macrophages caused by mycobacteria isolated from moldy houses. *Environmental Toxicology and Pharmacology*, **8**:237-244.

Huttunen K, Jussila J, Hirvonen MR, Iivanainen E, Katila ML. 2001. Comparison of mycobacteria-induced cytotoxicity and inflammatory responses in human and mouse cell lines. *Inhalation Toxicology*, **13**:977-991.

Huttunen K. 2003. Inflammatory and Cytotoxic Potential of Selected Moldy House Microbes *in vitro*. In: Publications of the National Public Health Institute A10. Kuopio, Finland: National Public Health Institute; p. 75.

Huttunen K, Hyvärinen A, Nevalainen A, Komulainen H, Hirvonen MR. 2003. Production of proinflammatory mediators by indoor air bacteria and fungal spores in mouse and human cell lines. *Environmental Health Perspectives*, **111**:85-92.

Huttunen K, Pelkonen J, Nielsen KF, Nuutinen U, Jussila J, Hirvonen MM. 2004. Synergistic interaction in simultaneous exposure to *Streptomyces californicus* and *Stachybotrys chartarum*. *Environmental Health Perspectives*, **112**:659-665.

Huws SA, Morley RJ, Jones MV, Brown MRW, Smith AW. 2008. Interactions of some common pathogenic bacteria with *Acanthamoeba polyphaga*. *FEMS Microbiology Letters*, **282**:258-265.

Hyvärinen A. 2002. Characterizing Moisture Damaged Buildings - Environmental and Biological Monitoring. In: Publications of the National Public Health Institute A8. Kuopio, Finland: National Public Health Institute; p. 121.

Hyvärinen A, Meklin T, Vepsäläinen A, Nevalainen A. 2002. Fungi and actinobacteria in moisture-damaged building materials - concentrations and diversity. *International Biodeterioration & Biodegradation*, **49**:27-37.

Immonen J, Meklin T, Taskinen T, Nevalainen A, Korppi M. 2001. Skin-prick test findings in students from moisture- and mould-damaged schools: a 3-year follow-up study. *Pediatric Allergy and Immunology*, **12**:87-94.

Inglis TJ, Rigby P, Robertson TA, Dutton NS, Henderson M, Chang BJ. 2000. Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* species results in coiling phagocytosis, endamebic bacterial survival, and escape. *Infection and Immunity*, **68**:1681-1686.

IOM: Committee on Damp Indoor Spaces and Health - Board on Health Promotion and Disease Prevention IoM. 2004. Damp Indoor Spaces and Health. National Academies Press: Washington.

Jaakkola JJK, Hwang BF, Jaakkola N. 2005. Home dampness and molds, parental atopy, and asthma in childhood: A six-year population-based cohort study. *Environmental Health Perspectives*, **113**:357-361.

Jaakkola MS, Nordman H, Piipari R, Uitti J, Laitinen J, Karjalainen A, Hahtola P, Jaakkola JJK. 2002. Indoor dampness and molds and development of adult-onset asthma: A population-based incident case-control study. *Environmental Health Perspectives,* **110**:543-547.

Jensen T, Barnes WG, Meyers D. 1970. Axenic cultivation of large populations of *Acanthamoeba castellanii (*JBM). *Journal of Parasitology,* **56**:904-906.

Jeon KW. 1995. Bacterial endosymbiosis in amoebae. *Trends in Cell Biology,* **5**:137-140.

Jeon KW. 2004. Genetic and physiological interactions in the amoeba-bacteria symbiosis. *Journal of Eukaryotic Microbiology,* **51**:502-508.

Jeon TJ, Jeon KW. 2004. Gene switching in *Amoeba proteus* caused by endosymbiotic bacteria. *Journal of Cell Science,* **117**:535-543.

Jjemba PK. 2001. The interaction of protozoa with their potential prey bacteria in the rhizosphere. *Journal of Eukaryotic Microbiology,* **48**:320-324.

Jung SY, Matin A, Kim KS, Khan NA. 2007. The capsule plays an important role in *Escherichia coli* K1 interactions with *Acanthamoeba*. *International Journal for Parasitology,* **37**:417-423.

Jussila J, Ruotsalainen M, Komulainen H, Savolainen K, Nevalainen A, Hirvonen MR. 1999. *Streptomyces anulatus* from indoor air of moldy houses induce NO and IL-6 production in a human alveolar epithelial cell-line. *Environmental Toxicology and Pharmacology,* **7**:261-266.

Jussila J, Komulainen H, Huttunen K, Roponen M, Hälinen A, Hyvärinen A, Kosma VM, Pelkonen J, Hirvonen MR. 2001. Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building. *Toxicology and Applied Pharmacology,* **171**:61-69.

Jussila J, Komulainen H, Huttunen K, Roponen M, Iivanainen E, Torkko P, Kosma VM, Pelkonen J, Hirvonen MR. 2002a. *Mycobacterium terrae* isolated from indoor air of a moisture-damaged building induces sustained biphasic inflammatory response in mouse lungs. *Environmental Health Perspectives,* **110**:1119-1125.

Jussila J, Komulainen H, Kosma VM, Nevalainen A, Pelkonen J, Hirvonen MR. 2002b. Spores of *Aspergillus versicolor* isolated from indoor air of a moisture-damaged building provoke acute inflammation in mouse lungs. *Inhalation Toxicology,* **14**:1261-1277.

Jussila J, Pelkonen J, Kosma VM, Mäki-Paakkanen J, Komulainen H, Hirvonen MR. 2003. Systemic immunoresponses in mice after repeated exposure of lungs to spores of *Streptomyces californicus. Clinical and Diagnostic Laboratory Immunology,* **10**:30-37.

Kadlec V. 1978. The occurrence of amphizoic amebae in domestic animals. *Journal of Protozoology,* **25**:235-237.

Kahane S, Dvoskin B, Mathias M, Friedman MG. 2001. Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. *Applied and Environmental Microbiology,* **67**:4789-4795.

Kilburn KH. 2003. Indoor mold exposure associated with neurobehavioral and pulmonary impairment: A preliminary report. *Archives of Environmental Health,* **58**:390-398.

Kilpeläinen M, Terho EO, Helenius H, Koskenvuo M. 2001. Home dampness, current allergic diseases, and respiratory infections among young adults. *Thorax,* **56**:462-467.

King CH, Shotts EB, Jr., Wooley RE, Porter KG. 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Applied and Environmental Microbiology,* **54**:3023-3033.

Korpi A, Pasanen AL, Pasanen P. 1998. Volatile compounds originating from mixed microbial cultures on building materials under various humidity conditions. *Applied and Environmental Microbiology,* **64**:2914-2919.

Koskinen OM, Husman TM, Meklin TM, Nevalainen AI. 1999a. Adverse health effects in children associated with moisture and mold observations in houses. *International Journal of Environmental Health Research,* **9**:143-156.

Koskinen OM, Husman TM, Meklin TM, Nevalainen AI. 1999b. The relationship between moisture or mould observations in houses and the state of health of their occupants. *European Respiratory Journal,* **14**:1363-1367.

Kuiper MW, Wullings BA, Akkermans AD, Beumer RR, van der Kooij D. 2004. Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. *Applied and Environmental Microbiology,* **70**:6826-6833.

Kuiper MW, Valster RM, Wullings BA, Boonstra H, Smidt H, van der Kooij D. 2006. Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. *Applied and Environmental Microbiology,* **72**:5750-5756.

La Scola B, Raoult D. 1999. *Afipia felis* in hospital water supply in association with free-living amoebae. *Lancet,* **353**:1330.

La Scola B, Barrassi L, Raoult D. 2000. Isolation of new fastidious alpha Proteobacteria and *Afipia felis* from hospital water supplies by direct plating and amoebal co-culture procedures. *FEMS Microbiology Ecology,* **34**:129-137.

La Scola B, Mezi L, Weiller PJ, Raoult D. 2001. Isolation of *Legionella anisa* using an amoebic coculture procedure. *Journal of Clinical Microbiology,* **39**:365-366.

La Scola B, Raoult D. 2001. Survival of *Coxiella burnetii* within free-living amoeba *Acanthamoeba castellanii*. *Clinical Microbiology and Infection,* **7**:75-79.

La Scola B, Audic S, Robert C, Jungang L, de Lamballerie X, Drancourt M, Birtles R, Claverie JM, Raoult D. 2003. A giant virus in amoebae. *Science*, **299**:2033.

La Scola B, Birtles RJ, Greub G, Harrison TJ, Ratcliff RM, Raoult D. 2004a. *Legionella drancourtii* sp. nov., a strictly intracellular amoebal pathogen. *International Journal of Systematic and Evolutionary Microbiology*, **54**:699-703.

La Scola B, Barrassi L, Raoult D. 2004b. A novel alpha-Proteobacterium, *Nordella oligomobilis* gen. nov., sp. nov., isolated by using amoebal co-cultures. *Research in Microbiology*, **155**:47-51.

Lamothe J, Thyssen S, Valvano MA. 2004. *Burkholderia cepacia* complex isolates survive intracellularly without replication within acidic vacuoles of *Acanthamoeba polyphaga*. *Cellular Microbiology*, **6**:1127-1138.

Landers P, Kerr KG, Rowbotham TJ, Tipper JL, Keig PM, Ingham E, Denton M. 2000. Survival and growth of *Burkholderia cepacia* within the free-living amoeba *Acanthamoeba polyphaga*. *European Journal of Clinical Microbiology and Infectious Diseases*, **19**:121-123.

Larkin DFP, Kilvington S, Easty DL. 1990. Contamination of contact lens storage cases by *Acanthamoeba* and bacteria. *British Journal of Ophthalmology*, **74**:133-135.

Lawande RV. 1983. Recovery of soil amoebae from the air during the harmattan in Zaria, Nigeria. *Annals of Tropical Medicine and Parasitology*, **77**:45-49.

Lebbadi M, Valdivia E, Gálvez A, Martínez-Bueno M, Maqueda M. 1995. Cocultivation of the amoeba *Naegleria fowleri* and the amoebicin-producing strain *Bacillus licheniformis* M-4. *Applied and Environmental Microbiology*, **61**:1649-1652.

Lebow ST, Winandy JE. 1999. Effect of fire-retardant treatment on plywood pH and the relationship of pH to strength properties. *Wood Science and Technology*, **33**:285-298.

Lee JJ, Leedale GF, Bradbury P. 2000. An illustrated guide to the protozoa. Lawrence, Kansas, U. S. A: Society of Protozoologists.

Lehtonen M, Reponen T, Nevalainen A. 1993. Everyday activities and variation of fungal spore concentrations in indoor air. *International Biodeterioration & Biodegradation*, **31**:25-39.

Lekkla A, Sutthikornchai C, Bovornkitti S, Sukthana Y. 2005. Free-living ameba contamination in natural hot springs in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, **36**:S5-9.

Lorenzo-Morales J, Coronado-Alvarez N, Martinez-Carretero E, Maciver SK, Valladares B. 2007. Detection of four adenovirus serotypes within water-isolated strains of *Acanthamoeba* in the Canary Islands, Spain. *American Journal of Tropical Medicine and Hygiene*, **77**:753-756.

Lozano-Alarcón F, Bradley GA, Houser BS, Visvesvara GS. 1997. Primary amebic meningoencephalitis due to *Naegleria fowleri* in a South American tapir. *Veterinary Pathology,* **34**:239-243.

Luosujärvi RA, Husman TM, Seuri M, Pietikäinen MA, Pollari P, Pelkonen J, Hujakka HT, Kaipiainen-Seppänen OA, Aho K. 2003. Joint symptoms and diseases associated with moisture damage in a health center. *Clinical Rheumatology,* **22**:381-385.

Luster MI, Simeonova PP, Gallucci R, Matheson J. 1999. Tumor necrosis factor alpha and toxicology. *Critical Reviews in Toxicology,* **29**:491-511.

Ly NP, Soto-Quiros ME, Avila L, Hunninghake GM, Raby BA, Laskey D, Sylvia JS, Celedon JC. 2008. Paternal asthma, mold exposure, and increased airway responsiveness among children with asthma in Costa Rica. *Chest,* **133**:107-114.

Ly TM, Müller HE. 1990. Ingested *Listeria monocytogenes* survive and multiply in protozoa. *Journal of Medical Microbiology,* **33**:51-54.

Mackiewicz B. 1998. Study on exposure of pig farm workers to bioaerosols, immunologic reactivity and health effects. *Annals of Agricultural and Environmental Medicine,* **5**:169-175.

Mansour NS, Saoud AFA, Nashed NN, Youssef FG. 1991. Fresh-water amoebae from four aquatic sites in Egypt. *Journal of the Egyptian Society of Parasitology,* **21**:15-22.

Marciano-Cabral F, Han K, Powell E, Ferguson T, Cabral G. 2003. Interaction of an *Acanthamoeba* human isolate harboring bacteria with murine peritoneal macrophages. *Journal of Eukaryotic Microbiology,* **50 Suppl**:516-519.

Markkanen P. 2008. Immutoxic Responses Induced by *Streptomyces californicus* and *Stachybotrys chartarum* - The Role of Microbial Interactions. In: Publications of the National Public Health Institute KTL A27. Kuopio, Finland: National Public Health Institute; p. 78.

Marolda CL, Hauröder B, John MA, Michel R, Valvano MA. 1999. Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. *Microbiology,* **145**:1509-1517.

Martínez AJ, Sotelo-Avila C, García-Tamayo J, Morón JT, Willaert E, Stamm WP. 1977. Meningoencephalitis due to *Acanthamoeba* sp. Pathogenesis and clinico-pathological study. *Acta Neuropathologica,* **37**:183-191.

Matheson MC, Abramson MJ, Dharmage SC, Forbes AB, Raven JM, Thien FC, Walters EH. 2005. Changes in indoor allergen and fungal levels predict changes in asthma activity among young adults. *Clinical and Experimental Allergy,* **35**:907-913.

Mattana A, Serra C, Mariotti E, Delogu G, Fiori PL, Cappuccinelli P. 2006. *Acanthamoeba castellanii* promotion of in vitro survival and transmission of coxsackie B3 viruses. *Eukaryotic Cell,* **5**:665-671.

Mayes DF, Rogerson A, Marchant H, Laybourn-Parry J. 1997. Growth and consumption rates of bacterivorous Antarctic naked marine amoebae. *Marine Ecology Progress Series,* **160**:101-108.

McFadden GI, Gilson PR, Hofmann CJ, Adcock GJ, Maier UG. 1994. Evidence that an amoeba acquired a chloroplast by retaining part of an engulfed eukaryotic alga. *Proceedings of the National Academy of Sciences of the United States of America,* **91**:3690-3694.

Meklin T. 2002. Microbial exposure and health in schools - effects of moisture damage and renovation. In: Publications of the National Public Health Institute A13. Kuopio, Finland: National Public Health Institute; p. 83.

Meyer HW, Würtz H, Suadicani P, Valbjørn O, Sigsgaard T, Gyntelberg F. 2004. Molds in floor dust and building-related symptoms in adolescent school children. *Indoor Air,* **14**:65-72.

Michel R, Burghardt H, Bergmann H. 1995a. Acanthamoebae isolated from a highly contaminated drinking-water system of a hospital exhibited natural infections with *Pseudomonas aeruginosa*. *Zentralblatt fur Hygiene und Umweltmedizin,* **196**:532-544.

Michel R, Müller KD, Schmid EN. 1995b. *Ehrlichia*-like organisms (KSL_1) observed as obligate intracellular parasites of *Saccamoeba* species. *Endocytobiosis & Cell Research,* **11**:69-80.

Michel R, Hauröder B. 1997. Isolation of an Acanthamoeba strain with intracellular *Burkholderia pickettii* infection. *Zentralblatt fur Bakteriologie,* **285**:541-557.

Michel R, Hauröder B, Müller KD, Zöller L. 1999. An environmental *Naegleria* strain, unable to form cysts - turned out to harbour two different species of endocytobionts. *Endocytobiosis & Cell Research,* **13**:115-118.

Michel R, Schmid EN, Böker T, Hager DG, Müller KD, Hoffmann R, Seitz HM. 2000. *Vannella* sp. harboring Microsporidia-like organisms isolated from the contact lens and inflamed eye of a female keratitis patient. *Parasitology Research,* **86**:514-520.

Michel R, Steinert M, Zöller L, Hauröder B, Henning K. 2004. Free-living amoebae may serve as hosts for the *Chlamydia*-like bacterium *Waddlia chondrophila* isolated from an aborted bovine foetus. *Acta Protozoologica,* **43**:37-42.

Michel R, Müller KD, Zöller L, Walochnik J, Hartmann M, Schmid EN. 2005. Free-living amoebae serve as a host for the *Chlamydia*-like bacterium *Simkania negevensis*. *Acta Protozoologica,* **44**:113-121.

Miltner EC, Bermudez LE. 2000. *Mycobacterium avium* grown in *Acanthamoeba castellanii* is protected from the effects of antimicrobials. *Antimicrobial Agents and Chemotherapy,* **44**:1990-1994.

Mirelman D. 1987. Ameba-bacterium relationship in amebiasis. *Microbiological Reviews,* **51**:272-284.

Molmeret M, Horn M, Wagner M, Santic M, Abu Kwaik Y. 2005. Amoebae as training grounds for intracellular bacterial pathogens. *Applied and Environmental Microbiology,* **71**:20-28.

Moncada S, Palmer RM, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews,* **43**:109-142.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods,* **65**:55-63.

Mrva M. 2007. Observations on morphology of insufficiently known amoebae *Saccamoeba wellneri* Siemensma, 1987 and *Saccamoeba limna* Bovee, 1972 (Gymnamoebia: Hartmannellidae). *Biologia,* **62**:315-319.

Müller KD, Schmid EN, Michel R. 1999. Intracellular bacteria of Acanthamoebae resembling *Legionella* spp. turned out to be *Cytophaga* sp. *Zentralblatt fur Bakteriologie,* **289**:389-397.

Munson DA. 1992. Marine amebas from Georgia coastal surface waters. *Transactions of the American Microscopical Society*, **111**:360-364.

Murtoniemi T. 2003. Microbial growth on plasterboard and spore-induced cytotoxicity and inflammatory responses in vitro. In: Publications of the National Public Health Institute A13. Kuopio, Finland: National Public Health Institute; p. 72.

Murtoniemi T, Penttinen P, Nevalainen A, Hirvonen MR. 2005. Effects of microbial cocultivation on inflammatory and cytotoxic potential of spores. *Inhalation Toxicology,* **17**:681-693.

Myllykangas-Luosujärvi R, Seuri M, Husman T, Korhonen R, Pakkala K, Aho K. 2002. A cluster of inflammatory rheumatic diseases in a moisture-damaged office. *Clinical and Experimental Rheumatology,* **20**:833-836.

Napolitano JJ. 1982. Isolation of amoebae from edible mushrooms. *Applied and Environmental Microbiology,* **44**:255-257.

Neumeister B, Schöniger S, Faigle M, Eichner M, Dietz K. 1997. Multiplication of different *Legionella* species in Mono Mac 6 cells and in *Acanthamoeba castellanii*. *Applied and Environmental Microbiology,* **63**:1219-1224.

Nevalainen A. 1989. Bacterial aerosols in indoor air. In: Publications of the National Public Health Institute A3. Kuopio, Finland: National Public Health Institute.

Nevalainen A, Pasanen AL, Niininen M, Reponen T, Kalliokoski P, Jantunen MJ. 1991. The Indoor Air Quality in Finnish homes with mold problems. *Environment International,* **17**:299-302.

Nevalainen A, Seuri M. 2005. Of microbes and men. *Indoor Air,* **15**:58-64.

Nevin BJ, Broadley KJ. 2002. Nitric oxide in respiratory diseases. *Pharmacology and Therapeutics*, **95**:259-293.

Newsome AL, Baker RL, Miller RD, Arnold RR. 1985. Interactions between *Naegleria fowleri* and *Legionella pneumophila*. *Infection and Immunity*, **50**:449-452.

Newsome AL, Scott TM, Benson RF, Fields BS. 1998. Isolation of an amoeba naturally harboring a distinctive *Legionella* species. *Applied and Environmental Microbiology*, **64**:1688-1693.

Nielsen KF, Gravesen S, Nielsen PA, Andersen B, Thrane U, Frisvad JC. 1999. Production of mycotoxins on artificially and naturally infested building materials. *Mycopathologia*, **145**:43-56.

Nikulin M, Reijula K, Jarvis BB, Hintikka EL. 1996. Experimental lung mycotoxicosis in mice induced by *Stachybotrys atra*. *International Journal of Experimental Pathology*, **77**:213-218.

Nikulin M, Reijula K, Jarvis BB, Veijalainen P, Hintikka EL. 1997. Effects of intranasal exposure to spores of *Stachybotrys atra* in mice. *Fundamental and Applied Toxicology*, **35**:182-188.

Page FC. 1988. A new key to freshwater and soil gymnamoebae. Freshwater Biological Association: Ambleside, Cumbria.

Pagnier I, Raoult D, La Scola B. 2008. Isolation and identification of amoeba-resisting bacteria from water in human environment by using an *Acanthamoeba polyphaga* co-culture procedure. *Environmental Microbiology*, **10**:1135-1144.

Park JH, Cox-Ganser J, Rao C, Kreiss K. 2006. Fungal and endotoxin measurements in dust associated with respiratory symptoms in a water-damaged office building. *Indoor Air*, **16**:192-203.

Park M, Yun ST, Kim MS, Chun J, Ahn TI. 2004. Phylogenetic characterization of *Legionella*-like endosymbiotic X-bacteria in *Amoeba proteus*: a proposal for *'Candidatus* Legionella jeonii' sp. nov. *Environmental Microbiology*, **6**:1252-1263.

Parthasarathi K, Ranganathan LS, Anandi V, Zeyer J. 2007. Diversity of microflora in the gut and casts of tropical composting earthworms reared on different substrates. *Journal of Environment Biology*, **28**:87-97.

Pasanen AL, Niininen M, Kalliokoski P, Nevalainen A, Jantunen MJ. 1992. Airborne *Cladosporium* and other fungi in damp *versus* reference residences. *Atmospheric Environment Part B-Urban Atmosphere*, **26**:121-124.

Pavlík V. 2000. Water extraction of chloride, hydroxide and other ions from hardened cement pastes. *Cement and Concrete Research*, **30**:895-906.

Peat JK, Dickerson J, Li J. 1998. Effects of damp and mould in the home on respiratory health: a review of the literature. *Allergy*, **53**:120-128.

Pekkanen J, Hyvärinen A, Haverinen-Shaughnessy U, Korppi M, Putus T, Nevalainen A. 2007. Moisture damage and childhood asthma: a population-based incident case-control study. *European Respiratory Journal*, **29**:509-515.

Penttinen P, Pelkonen J, Huttunen K, Toivola M, Hirvonen MR. 2005a. Interactions between *Streptomyces californicus* and *Stachybotrys chartarum* can induce apoptosis and cell cycle arrest in mouse RAW264.7 macrophages. *Toxicology and Applied Pharmacology*, **202**:278-288.

Penttinen P, Huttunen K, Pelkonen J, Hirvonen MR. 2005b. The proportions of *Streptomyces californicus* and *Stachybotrys chartarum* in simultaneous exposure affect inflammatory responses in mouse RAW264.7 macrophages. *Inhalation Toxicology*, **17**:79-85.

Penttinen P, Pelkonen J, Huttunen K, Hirvonen MR. 2006. Co-cultivation of *Streptomyces californicus* and *Stachybotrys chartarum* stimulates the production of cytostatic compound(s) with immunotoxic properties. *Toxicology and Applied Pharmacology*, **217**:342-351.

Penttinen P, Tampio M, Mäki-Paakkanen J, Vähäkangas K, Pelkonen J, Hirvonen MR. 2007. DNA damage and p53 in RAW264.7 cells induced by the spores of co-cultivated *Streptomyces californicus* and *Stachybotrys chartarum*. *Toxicology*, **235**:92-102.

Piecková E, Jesenska Z. 1996. Filamentous microfungi in raw flax and cotton for textile industry and their ciliostatic activity on tracheal organ cultures in vitro. *Mycopathologia*, **134**:91-96.

Piecková E, Jesenska Z. 1998. Molds on house walls and the effect of their chloroform-extractable metabolites on the respiratory cilia movement of one-day-old chicks in vitro. *Folia Microbiologica*, **43**:672-678.

Piecková E, Wilkins K. 2004. Airway toxicity of house dust and its fungal composition. *Annals of Agricultural and Environmental Medicine*, **11**:67-73.

Pirhonen I, Nevalainen A, Husman T, Pekkanen J. 1996. Home dampness, moulds and their influence on respiratory infections and symptoms in adults in Finland. *European Respiratory Journal*, **9**:2618-2622.

Pohland AE. 1993. Mycotoxins in review. *Food Additives and Contaminants*, **10**:17-28.

Purokivi A, Hirvonen MR, Roponen M, Randell J, Vahteristo A, Tukiainen H. 2002. Comparison of inflammatory elements in nasal lavage and induced sputum following occupational exposure to moldy-building microbes. *Inhalation Toxicology*, **14**:653-662.

Purokivi MK, Hirvonen MR, Randell JT, Roponen MH, Meklin TM, Nevalainen AI, Human TM, Tukiainen HO. 2001. Changes in pro-inflammatory cytokines in association with exposure to moisture-damaged building microbes. *European Respiratory Journal*, **18**:951-958.

Pylkkänen L, Gullsten H, Majuri ML, Andersson U, Vanhala E, Määttä J, Meklin T, Hirvonen MR, Alenius H, Savolainen K. 2004. Exposure to *Aspergillus fumigatus* spores induces chemokine expression in mouse macrophages. *Toxicology,* **200**:255-263.

Qvarnström Y, Visvesvara GS, Sriram R, da Silva AJ. 2006. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. *Journal of Clinical Microbiology,* **44**:3589-3595.

Raoult D, La Scola B, Birtles R. 2007. The discovery and characterization of Mimivirus, the largest known virus and putative pneumonia agent. *Clinical Infectious Diseases,* **45**:95-102.

Reiman M, Haatainen S, Kallunki H, Kujanpää L, Laitinen S, Rautiala S. 1999. The characteristics of the dilution and direct plating methods for the determination of microbial flora and concentrations in building materials. *Proceedings of the 8th International Conference on Indoor Air Quality and Climate,* **4**:891-896.

Reponen T, Hyvärinen A, Ruuskanen J, Raunemaa T, Nevalainen A. 1994. Comparison of concentrations and size distributions of fungal spores in buildings with and without mold problems. *Journal of Aerosol Science,* **25**:1595-1603.

Rideout BA, Gardiner CH, Stalis IH, Zuba JR, Hadfield T, Visvesvara GS. 1997. Fatal infections with *Balamuthia mandrillaris* (a free-living amoeba) in gorillas and other Old World primates. *Veterinary Pathology,* **34**:15-22.

Rintala H, Pitkäranta M, Toivola M, Paulin L, Nevalainen A. 2008. Diversity and seasonal dynamics of bacterial community in indoor environment. *BMC Microbiology,* **8**:56.

Rivera F, Ramirez P, Vilaclara G, Robles E, Medina F. 1983. A survey of pathogenic and free-living amoebae inhabiting swimming pool water in Mexico City. *Environmental Research,* **32**:205-211.

Rivera F, Roy-Ocotla G, Rosas I, Ramirez E, Bonilla P, Lares F. 1987. Amoebae isolated from the atmosphere of Mexico City and environs. *Environmental Research,* **42**:149-154.

Rivera F, Lares F, Gallegos E, Ramirez E, Bonilla P, Calderon A, Martinez JJ, Rodriguez S, Alcocer J. 1989. Pathogenic amoebae in natural thermal waters of three resorts of Hidalgo, Mexico. *Environ Res,* **50**:289-295.

Rivera F, Ramirez E, Bonilla P, Calderon A, Gallegos E, Rodriguez S, Ortiz R, Zaldivar B, Ramirez P, Duran A. 1993. Pathogenic and free-living amoebae isolated from swimming pools and physiotherapy tubs in Mexico. *Environmental Research,* **62**:43-52.

Rivière D, Szczebara FM, Berjeaud JM, Frere J, Hechard Y. 2006. Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. *Journal of Microbiological Methods,* **64**:78-83.

Robinson BS, De Jonckheere JF, Dobson PJ. 2007. Two new *Tetramitus* species (Heterolobosea, Vahlkampfiidae) from cold aquatic environments. *European Journal of Protistology,* **43**:1-7.

Rodríguez-Zaragoza S. 1994. Ecology of free-living amoebae. *Critical Reviews in Microbiology,* **20**:225-241.

Rodríguez-Zaragoza S, Mayzlish E, Steinberger Y. 2005. Seasonal changes in free-living amoeba species in the root canopy of *Zygophyllum dumosum* in the Negev Desert, Israel. *Microbial Ecology,* **49**:134-141.

Rohr U, Weber S, Michel R, Selenka F, Wilhelm M. 1998. Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance. *Applied and Environmental Microbiology*, **64**:1822-1824.

Rønn R, McCaig AE, Griffiths BS, Prosser JI. 2002. Impact of protozoan grazing on bacterial community structure in soil microcosms. *Applied and Environmental Microbiology,* **68**:6094-6105.

Roponen M, Toivola M, Meklin T, Ruotsalainen M, Komulainen H, Nevalainen A, Hirvonen MR. 2001a. Differences in inflammatory responses and cytotoxicity in RAW264.7 macrophages induced by *Streptomyces anulatus* grown on different building materials. *Indoor Air,* **11**:179-184.

Roponen M, Kiviranta J, Seuri M, Tukiainen H, Myllykangas-Luosujärvi R, Hirvonen MR. 2001b. Inflammatory mediators in nasal lavage, induced sputum and serum of employees with rheumatic and respiratory disorders. *European Respiratory Journal,* **18**:542-548.

Roponen M, Toivola M, Alm S, Nevalainen A, Jussila J, Hirvonen MR. 2003. Inflammatory and cytotoxic potential of the airborne particle material assessed by nasal lavage and cell exposure methods. *Inhalation Toxicology,* **15**:23-38.

Rotter BA, Prelusky DB, Pestka JJ. 1996. Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health,* **48**:1-34.

Rude RA, Jackson GJ, Bier JW, Sawyer TK, Risty NG. 1984. Survey of fresh vegetables for nematodes, amoebae, and *Salmonella*. *Journal - Association of Official Analytical Chemists,* **67**:613-615.

Sadaka HA, El-Nassery SF, Abou Samra LM, Awadalla HN. 1994. Isolation and identification of free-living amoebae from some water sources in Alexandria. *Journal of the Egyptian Society of Parasitology,* **24**:247-257.

Saeed A, Abd H, Edvinsson B, Sandström G. 2007. *Vibrio cholerae-Acanthamoeba castellanii* interaction showing endosymbiont-host relation. *Symbiosis,* **44**:153-158.

Salo PM, Jiang X, Johnson CA, Yan L, Avol EL, Gong J, London SJ. 2004. Indoor allergens, asthma, and asthma-related symptoms among adolescents in Wuhan, China. *Annals of Epidemiology,* **14**:543-550.

Salonen H, Lappalainen S, Lindroos O, Harju R, Reijula K. 2007. Fungi and bacteria in mould-damaged and non-damaged office environments in a subarctic climate. *Atmospheric Environment,* **41**:6797-6807.

Samson RA, Flannigan B, Flannigan ME, Verhoeff AP, Adan OCG, Hoekstra ES. 1994. Recommendations. In: Health implications of fungi in indoor environments. (Ed Samson RA, Flannigan B, Flannigan ME, Verhoeff AP, Adan OCG, Hoekstra ES). Amsterdam, the Netherlands: Elsevier Science B. V.; p. 529-538.

Santos P, Pinhal I, Rainey FA, Empadinhas N, Costa J, Fields B, Benson R, Veríssimo A, da Costa MS. 2003. Gamma-proteobacteria *Aquicella lusitana* gen. nov., sp. nov., and *Aquicella siphonis* sp. nov. infect protozoa and require activated charcoal for growth in laboratory media. *Applied and Environmental Microbiology,* **69**:6533-6540.

Sawyer TK, Visvesvara GS, Harke BA. 1977. Pathogenic amoebas from brackish and ocean sediments, with a description of *Acanthamoeba hatchetti*, n. sp. *Science,* **196**:1324-1325.

Sawyer TK. 1989. Free-living pathogenic and nonpathogenic amoebae in Maryland soils. *Applied and Environmental Microbiology,* **55**:1074-1077.

Schild M, Gianinazzi C, Gottstein B, Müller N. 2007. PCR-based diagnosis of *Naegleria* sp. infection in formalin-fixed and paraffin-embedded brain sections. *Journal of Clinical Microbiology,* **45**:564-567.

Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, Fuerst PA, Byers TJ. 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. *Journal of Clinical Microbiology,* **39**:1903-1911.

Schuster FL. 2002. Cultivation of pathogenic and opportunistic free-living amebas. *Clinical Microbiology Reviews,* **15**:342-354.

Schuster FL, Dunnebacke TH, Booton GC, Yagi S, Kohlmeier CK, Glaser C, Vugia D, Bakardjiev A, Azimi P, Maddux-Gonzalez M, Martinez AJ, Visvesvara GS. 2003. Environmental isolation of *Balamuthia mandrillaris* associated with a case of amebic encephalitis. *Journal of Clinical Microbiology,* **41**:3175-3180.

Seal D, Stapleton F, Dart J. 1992. Possible environmental sources of *Acanthamoeba* spp in contact lens wearers. *British Journal of Ophthalmology,* **76**:424-427.

Sedgwick JB, Menon I, Gern JE, Busse WW. 2002. Effects of inflammatory cytokines on the permeability of human lung microvascular endothelial cell monolayers and differential eosinophil transmigration. *Journal of Allergy and Clinical Immunology,* **110**:752-756.

Seuri M, Paldanius M, Leinonen M, Roponen M, Hirvonen MR, Saikku P. 2005. *Chlamydophila pneumoniae* antibodies in office workers with and without inflammatory rheumatic diseases in a moisture-damaged building. *European Journal of Clinical Microbiology and Infectious Diseases,* **24**:236-237.

Shadrach WS, Rydzewski K, Laube U, Holland G, Ozel M, Kiderlen AF, Flieger A. 2005. *Balamuthia mandrillaris*, free-living ameba and opportunistic agent of encephalitis, is a potential host for *Legionella pneumophila* bacteria. *Applied and Environmental Microbiology,* **71**:2244-2249.

Shenassa ED, Daskalakis C, Liebhaber A, Braubach M, Brown M. 2007. Dampness and mold in the home and depression: An examination of mold-related illness and perceived control of one's home as possible depression pathways. *American Journal of Public Health,* **97**:1893-1899.

Shoff ME, Rogerson A, Kessler K, Schatz S, Seal DV. 2008. Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water. *Journal of Water and Health,* **6**:99-104.

Simoni M, Lombardi E, Berti G, Rusconi F, La Grutta S, Piffer S, Petronio MG, Galassi C, Forastiere F, Viegi G, Grp S-C. 2005. Mould/dampness exposure at home is associated with respiratory disorders in Italian children and adolescents: the SIDRIA-2 Study. *Occupational and Environmental Medicine,* **62**:616-622.

Skriwan C, Fajardo M, Hägele S, Horn M, Wagner M, Michel R, Krohne G, Schleicher M, Hacker J, Steinert M. 2002. Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. *International Journal of Medical Microbiology,* **291**:615-624.

Smirnov A, Brown S. 2004. Guide to the methods of study and identification of soil gymnamoebae. *Protistology,* **3**:148-190.

Snelling WJ, McKenna JP, Lecky DM, Dooley JSG. 2005. Survival of *Campylobacter jejuni* in waterborne protozoa. *Applied and Environmental Microbiology,* **71**:5560-5571.

Solomon JM, Rupper A, Cardelli JA, Isberg RR. 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. *Infection and Immunity,* **68**:2939-2947.

Solomon JM, Leung GS, Isberg RR. 2003. Intracellular replication of *Mycobacterium marinum* within *Dictyostelium discoideum*: efficient replication in the absence of host coronin. *Infection and Immunity,* **71**:3578-3586.

Spice WM, Ackers JP. 1992. The effect of axenic versus xenic culture conditions on the total and secreted proteolytic activity of *Entamoeba histolytica* strains. *Archives of Medical Research,* **23**:91-93.

Stark H, Roponen M, Purokivi M, Randell J, Tukiainen H, Hirvonen MR. 2006. *Aspergillus fumigatus* challenge increases cytokine levels in nasal lavage fluid. *Inhalation Toxicology,* **18**:1033-1039.

Stauffer W, Ravdin JI. 2003. *Entamoeba histolytica*: an update. *Current Opinion in Infectious Diseases,* **16**:479-485.

Steenbergen JN, Nosanchuk JD, Malliaris SD, Casadevall A. 2003. *Cryptococcus neoformans* virulence is enhanced after growth in the genetically malleable host *Dictyostelium discoideum*. *Infection and Immunity,* **71**:4862-4872.

Steinert M, Birkness K, White E, Fields B, Quinn F. 1998. *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology,* **64**:2256-2261.

STM. 2003. Asumisterveysohje. Asuntojen ja muiden oleskelutilojen fysikaaliset, kemialliset ja mikrobiologiset tekijät. Sosiaali- ja terveysministeriön oppaita 2003:1: Helsinki.

Stockmann-Juvala H, Naarala J, Loikkanen J, Vahakangas K, Savolainen K. 2006. Fumonisin B1-induced apoptosis in neuroblastoma, glioblastoma and hypothalamic cell lines. *Toxicology,* **225**:234-241.

Stothard DR, Schroeder-Diedrich JM, Awwad MH, Gast RJ, Ledee DR, Rodriguez-Zaragoza S, Dean CL, Fuerst PA, Byers TJ. 1998. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *Journal of Eukaryotic Microbiology,* **45**:45-54.

Sykes DE, Band RN. 1985. Polyphenol oxidase produced during encystation of *Acanthamoeba castellanii*. *Journal of Protozoology,* **32**:512-517.

Sykora JL, Keleti G, Martinez AJ. 1983. Occurrence and pathogenicity of *Naegleria fowleri* in artificially heated waters. *Applied and Environmental Microbiology,* **45**:974-979.

Taskinen T, Meklin T, Nousiainen M, Husman T, Nevalainen A, Korppi M. 1997. Moisture and mould problems in schools and respiratory manifestations in schoolchildren: clinical and skin test findings. *Acta Paediatrica,* **86**:1181-1187.

Temprano J, Becker BA, Hutcheson PS, Knutsen AP, Dixit A, Slavin RG. 2007. Hypersensitivity pneumonitis secondary to residential exposure to *Aureobasidium pullulans* in 2 siblings. *Annals of Allergy, Asthma, and Immunology,* **99**:562-566.

Tezcan-Merdol D, Ljungstrom M, Winiecka-Krusnell J, Linder E, Engstrand L, Rhen M. 2004. Uptake and replication of *Salmonella enterica* in *Acanthamoeba rhysodes*. *Applied and Environmental Microbiology,* **70**:3706-3714.

Tham KW, Zuraimi MS, Koh D, Chew FT, Ooi PL. 2007. Associations between home dampness and presence of molds with asthma and allergic symptoms among young children in the tropics. *Pediatric Allergy and Immunology,* **18**:418-424.

Thom S, Warhurst D, Drasar BS. 1992. Association of *Vibrio cholerae* with fresh water amoebae. *Journal of Medical Microbiology,* **36**:303-306.

Thomas V, Casson N, Greub G. 2006. *Criblamydia sequanensis*, a new intracellular *Chlamydiales* isolated from Seine river water using amoebal co-culture. *Environmental Microbiology*, **8**:2125-2135.

Todoriki M, Urabe I. 2006. Induced symbiosis: distinctive *Escherichia coli* - *Dictyostelium discoideum* transferable co-cultures on agar. *Symbiosis*, **42**:135-139.

Tomov AT, Tsvetkova ED, Tomova IA, Michailova LI, Kassovski VK. 1999. Persistence and multiplication of obligate anaerobe bacteria in amebae under aerobic conditions. *Anaerobe*, **5**:19-23.

Tsai FC, Macher JM. 2005. Concentrations of airborne culturable bacteria in 100 large US office buildings from the BASE study. *Indoor Air*, **15**:71-81.

Tsai FC, Macher JM, Hung YY. 2007. Biodiversity and concentrations of airborne fungi in large US office buildings from the BASE study. *Atmospheric Environment*, **41**:5181-5191.

Tsvetkova N, Schild M, Panaiotov S, Kurdova-Mintcheva R, Gottstein B, Walochnik J, Aspöck H, Lucas MS, Müller N. 2004. The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitology Research*, **92**:405-413.

Turner NA, Russell AD, Furr JR, Lloyd D. 2004. Resistance, biguanide sorption and biguanide-induced pentose leakage during encystment of *Acanthamoeba castellanii*. *Journal of Applied Microbiology*, **96**:1287-1295.

Tyndall RL, Lyle MM, Ironside KS. 1987. The presence of free-living amoebae in portable and stationary eye wash stations. *American Industrial Hygiene Association Journal*, **48**:933-934.

van Assendelft A, Forsen KO, Keskinen H, Alanko K. 1979. Humidifier-associated extrinsic allergic alveolitis. *Scandinavian Journal of Work, Environment and Health*, **5**:35-41.

Van Strien RT, Verhoeff AP, Brunekreef B, Van Wijnen JH. 1994. Mite antigen in house dust: relationship with different housing characteristics in The Netherlands. *Clinical and Experimental Allergy*, **24**:843-853.

Verhoeff AP, Hoekstra ES, Samson RA, Brunekreef B, van Wijnen JH. 1994. Fungal propagules in house dust: comparison of analytical methods. In: Health Implications of Fungi in Indoor Environments. (Ed Samson RA, Flannigan B, Flannigan ME, Verhoeff AP, Adan OCG, Hoekstra ES). Amsterdam, The Netherlands: Elsevier Science B. V.; p. 49-63.

Vesaluoma M, Kalso S, Jokipii L, Warhurst D, Ponka A, Tervo T. 1995. Microbiological quality in Finnish public swimming pools and whirlpools with special reference to free living amoebae: a risk factor for contact lens wearers? *British Journal of Ophthalmology*, **79**:178-181.

Visvesvara GS, Jones DB, Robinson NM. 1975. Isolation, identification, and biological characterization of *Acanthamoeba polyphaga* from a human eye. *American Journal of Tropical Medicine and Hygiene,* **24**:784-790.

Visvesvara GS, Schuster FL, Martinez AJ. 1993. *Balamuthia mandrillaris*, n. g., n. sp., agent of amebic meningoencephalitis in humans and other animals. *Journal of Eukaryotic Microbiology*, **40**:504-514.

Visvesvara GS, Booton GC, Kelley DJ, Fuerst P, Sriram R, Finkelstein A, Garner MM. 2007. In vitro culture, serologic and molecular analysis of *Acanthamoeba* isolated from the liver of a keel-billed toucan (*Ramphastos sulfuratus*). *Veterinary Parasitology*, **143**:74-78.

Vodkin MH, Howe DK, Visvesvara GS, McLaughlin GL. 1992. Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. *Journal of Protozoology*, **39**:378-385.

VreekenBuijs MJ, Geurs M, deRuiter PC, Brussaard L. 1997. The effects of bacterivorous mites and amoebae on mineralization in a detrital based below-ground food web; microcosm experiment and simulation of interactions. *Pedobiologia,* **41**:481-493.

Wagner Y, Noack B, Hoffmann T, Jacobs E, Lück PC. 2006. Periodontopathogenic bacteria multiply in the environmental amoeba *Acanthamoeba castellani*. *International Journal of Hygiene and Environmental Health*, **209**:535-539.

Walochnik J, Picher O, Aspöck C, Ullmann M, Sommer R, Aspöck H. 1998. Interactions of "Limax amoebae" and gram-negative bacteria: experimental studies and review of current problems. *Tokai Journal of Experimental and Clinical Medicine,* **23**:273-278.

Walochnik J, Obwaller A, Aspöck H. 2001. Immunological inter-strain crossreactivity correlated to 18S rDNA sequence types in *Acanthamoeba* spp. *International Journal for Parasitology*, **31**:163-167.

Walochnik J, Müller KD, Aspöck H, Michel R. 2005. An endocytobiont harbouring *Naegleria* strain identified as *N. clarki* de Jonckheere, 1994. *Acta Protozoologica,* **44**:301-310.

Weekers PH, De Jonckheere JF. 1997. Differences in isoenzyme patterns of axenically and monoxenically grown *Acanthamoeba* and *Hartmannella*. *Antonie Van Leeuwenhoek,* **71**:231-237.

Whan L, Grant IR, Rowe MT. 2006. Interaction between *Mycobacterium avium* subsp. paratuberculosis and environmental protozoa. *BMC Microbiology*, **6**:63.

WHO. 2003. Chapter 5: Free-living microorganisms. In: Guidelines for safe recreational water environments. Volume 1. Coastal and fresh waters. Geneva: World Health Organization; p. 112-117.

Winiecka-Krusnell J, Wreiber K, von Euler A, Engstrand L, Linder E. 2002. Free-living amoebae promote growth and survival of *Helicobacter pylori*. *Scandinavian Journal of Infectious Diseases,* **34**:253-256.

Xinyao L, Miao S, Yonghong L, Yin G, Zhongkai Z, Donghui W, Weizhong W, Chencai A. 2006. Feeding characteristics of an amoeba (Lobosea: Naegleria) grazing upon cyanobacteria: food selection, ingestion and digestion progress. *Microbial Ecology,* **51**:315-325.

Xuan YH, Yu HS, Jeong HJ, Seol SY, Chung DI, Kong HH. 2007. Molecular characterization of bacterial endosymbionts of *Acanthamoeba* isolates from infected corneas of Korean patients. *Korean Journal of Parasitology,* **45**:1-9.

Yagita K, Matias RR, Yasuda T, Natividad FF, Enriquez GL, Endo T. 1995. *Acanthamoeba* sp. from the Philippines: electron microscopy studies on naturally occurring bacterial symbionts. *Parasitology Research,* **81**:98-102.

Yu HS, Jeong HJ, Hong YC, Seol SY, Chung DI, Kong HH. 2007. Natural occurrence of *Mycobacterium* as an endosymbiont of *Acanthamoeba* isolated from a contact lens storage case. *Korean Journal of Parasitology,* **45**:11-18.

Zimmermann H. 1997. The microbial community on aggregates in the Elbe Estuary, Germany. *Aquatic Microbial Ecology,* **13**:37-46.