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Amoebae and other protozoa in material samples from moisture-damaged buildings ☆

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Abstract

Mold growth in buildings has been shown to be associated with adverse health effects. The fungal and bacterial growth on moistened building materials has been studied, but little attention has been paid to the other organisms spawning in the damaged materials. We examined moist building materials for protozoa, concentrating on amoebae. Material samples ($n = 124$) from moisture-damaged buildings were analyzed for amoebae, fungi, and bacteria. Amoebae were detected in 22% of the samples, and they were found to favor cooccurrence with bacteria and the fungi *Acremonium* spp., *Aspergillus versicolor*, *Chaetomium* spp., and *Trichoderma* spp. In addition, 11 seriously damaged samples were screened for other protozoa. Ciliates and flagellates were found in almost every sample analyzed. Amoebae are known to host pathogenic bacteria, such as chlamydiae, legionellae, and mycobacteria and they may have a role in the complex of exposure that contributes to the health effects associated with moisture damage in buildings.

1. Introduction

It has been epidemiologically shown that moisture damage with consequent microbial growth in buildings and the health problems of residents are strongly associated (Verhoeff and Burge, 1997; Peat et al., 1998; Bornehag et al., 2001; and others). Health problems include mainly respiratory symptoms and diseases, but unspecific symptoms, such as fever, headache, and muscle pain, have also been reported (Verhoeff and Burge, 1997; Husman, 1996). The causative agents for the health effects are still mostly unknown and the exposure in fact consists of a complex mixture of agents and substances. In addition to fungi and bacteria, the growth of other organisms, such as

mites, may also be related to water damage (Korsgaard, 1983). Dampness in the building materials may facilitate emissions of microbial toxins and VOCs from both microbial growth and chemical degradation of building materials (Pohland, 1993; Korpi et al., 1998; Batterman, 1995). All of these may contribute to the exposure caused by moisture damage.

Among the biological agents in indoor environments are also organisms connected to microbial growth. Species of amoebae, flagellates, and ciliates are ubiquitous in natural environments containing water (Storer et al., 1979). Many of these organisms can form resistant forms, e.g., cysts, in unfavorable environmental conditions (Rogerson and Patterson, 2000). Protozoa feed on bacteria, fungi, yeasts, algae, or even other protozoa (Storer et al., 1979). Thus, some amoebae and other protozoa should be able to grow on moistened building materials.

Many of the free-living amoebae are facultative or obligate pathogens to humans and animals (Rogerson and Patterson, 2000). In particular, some species of the genera *Naegleria*, *Acanthamoeba*, and *Balamuthia* may

invade humans (Page, 1976; Visvesvara, 1999). Species of *Acanthamoeba* and *Naegleria* have been shown to harbor several species of bacteria as natural endosymbionts. Many of these bacteria are unculturable obligate endosymbionts and have not been recognized (Winiecka-Krusnell and Linder, 2001), but also Legionellae, *Pseudomonas aeruginosa*, bacteria resembling rickettsiae, erlichiae, and chlamydiae, among others, have been found inside environmental amoebae (Rowbotham, 1980; Michel et al., 1995a, b; Fritsche et al., 1999; Amann et al., 1997). Thus, amoebae can offer a sheltered site for bacteria otherwise unable to survive in the building material matrix.

Many other bacteria have been shown in vitro to be able to survive and even replicate inside of amoebae; for example, the human pathogens *Chlamydia pneumoniae*, *Burkholderia cepacia* complex, and *Mycobacterium avium* (Essig et al., 1997; Marolda et al., 1999; Cirillo et al., 1997). These bacteria are associated with respiratory symptoms that are also common symptoms among occupants of moisture-damaged buildings (Cirillo et al., 1997; Husman, 1996). Of the bacteria, *M. avium*, among other *Mycobacterium* strains, has been isolated in the indoor air of a moisture- and mold-damaged building during renovation and from material samples obtained from a water-damaged building (Rautiala et al., 1995; Andersson et al., 1997).

Even though the association between house dampness and numerous symptoms has been extensively researched, the exact roles of various agents in the development of the adverse health outcomes remain poorly understood. The fungal contamination and, to some extent, bacteria of moisture-damaged building materials have been previously studied, but little attention has been paid to other organisms inhabiting the materials. At present, there is no evident and known mechanism by which the fungi or environmental bacteria alone could be the agents causing the health effects. In the search for sources of agents behind the reported symptoms, we wanted to reveal what other organisms could be found in the building materials.

The aims of this study were to investigate whether amoebae occur in moisture-damaged building materials and to make a preliminary survey of the types of other protozoa in these habitats.

2. Materials and methods

Samples of building materials ($n = 124$) were analyzed for amoebae, fungi, and bacteria (including actinomycetes). The samples used in this study were taken from moisture-damaged buildings. Most samples selected for the current study were visibly damaged, but less damaged samples from the same buildings were also analyzed. The samples included several different types of construction materials: mineral insulation (42%), wood (17%), concrete and ceramic products (10%), paints and glues (3%), gypsum board and other nonwood boards (10%), wallpaper (6%), assorted plastics (2%), and others (10%). After the microbial analyses, the samples were oven dried for 1 h at 100°C to determine the water content of the material.

2.1. Amoebae

The presence of amoebae was determined according to the method described by Newsome et al. (1998). Briefly, nonnutrient agar plates were streaked in an "X" configuration with heat-killed *Escherichia coli* (American Type Culture Collection, Strain 25922). A small piece of the sample (ca. $1 \times 1 \, cm^2$) was placed in the center of the *E. coli* lines on the plate and the plates were incubated at 25°C for 48–72 h. Plates were examined microscopically along the *E. coli* lines and around the material sample with magnifications of 100–400 for the presence of amoebae. The amount of trophozoites and cysts on each plate was originally estimated with a five-step classification: not present, 0; up to 10 trophozoites and cysts, 1; up to 100, 2; up to 1000, 3; more than 1000, 4. However, for statistical analyses these original amoebic data were classified into three categories, as described in Table 1.

2.2. Fungi and bacteria

The samples were cultivated either by direct plating ($n = 75$) or by dilution plating ($n = 49$) as previously described (Reiman et al., 1999; Hyvärinen et al., 2002). In the direct plating, a portion of the roughly homogenized material sample was collected with a standard-size oval spoon (sized $10 \times 20 \times 2 \, mm^3$, approximately

Table 1
Classification key for amounts of amoebae, bacteria, and fungi

Class	Amoebae (trophozoites and cysts)	Bacteria		Fungi	
		Dilution plating (cfu/g)	Direct plating (description)	Dilution plating (cfu/g)	Direct plating (description)
0	None	<10,000	Little or no growth	<10,000	Little or no growth
1	1–1000	10,000–1,000,000	Intermediate growth	10,000–100,000	Intermediate growth
2	>1000	>1,000,000	Abundant growth	>100,000	Abundant growth

0.5 mL). These subsamples were dispersed evenly on the agar media. Samples were cultivated on four types of agar plates: fungi were grown on Rose bengal malt extract agar (Hagem), dichloran glyserol agar (DG18), and malt extract agar (MEA), and bacteria were grown 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 min, respectively. Aliquots (100 μL) of serial dilutions were spread evenly on MEA, DG18, and TYG media (Hyvärinen et al., 2002).

All of the samples were incubated in the dark for 5 days (bacteria), 7 days (fungi), or 14 days (actinomycetes) at 20–25°C. After the incubation, colonies were counted. Fungi were identified to the genus level by optical microscopy, except for *Aspergillus versicolor*, *A. fumigatus*, *A. terreus*, and *A. niger*, which were identified separately. In order to combine the data from both types of microbial cultivation (direct or dilution plating), data also were classified into three groups on the basis of colony counts, as described in Table 1.

2.3. Other protozoa

Analysis for other fauna was conducted after the fungal and bacterial analyses. Severely damaged samples (*n* = 11) were stored at 7°C in air-proofed plastic bags. A piece of the material was put on a nonnutrient agar plate, and 0.5 mL of sterilized spring water was added to the top of the sample. Water was added to aid the movement of the protozoans and thus make them visible under a microscope. Samples were incubated for 48 h at 25°C. After the incubation, samples were observed on a stereomicroscope with magnifications of 100–400. If protozoa were found, the samples were also observed with a magnification of 1000 on object glass. The organisms found were identified based on their morphological characteristics.

2.4. Statistical methods

All data were analyzed with SPSS Version 10.1.3 using Fishers' exact test, a Kruskal–Wallis test, or a Mann–Whitney test for nonparametric independent samples. Principal component analysis was used to verify that the association of certain fungal genera with the presence of amoebae was not the result of interfungal correlations (data not shown). The data from microbial and amoebic analyses were split into two categories, present and not present, for determining the possible cooccurrence of amoebae with fungi and bacteria. The association of amounts of amoebae and fungi or bacteria was analyzed using the three-step classification for all microbes.

3. Results

The samples varied from extremely damaged to almost intact material. Of the 124 samples, amoebae were detected in 27 cases (≈ 22%). Amoebae were detected mostly in the more mold-damaged materials, but not at all in the samples with no or little microbial contamination. Water content affected both the occurrence of amoebae and the number of amoebae significantly (Kruskal–Wallis, $P = 0.000$ for both). The average water content per dry weight (kg/kg) for samples positive for amoebae was 30.9% and for those negative for amoebae was 3.5%. All of the samples with a greater than water content 38% were positive for amoebae. The lowest water content of a sample still positive for amoebae was 0.4% for mineral insulation material.

There were significant associations between the occurrence of the amoebae and certain fungi and bacteria. Samples with amoebae were always positive for bacteria (Fisher's exact, $P = 0.039$), and amoebae favored cooccurrence with actinomycetes ($P = 0.000$) and other bacteria ($P = 0.040$). Samples with amoebae were not necessarily positive for fungi (total culturable fungi). However, at the genus level significant associations with fungi and amoebae were detected. Amoebae favored *Acremonium* ($P = 0.008$), *A. versicolor* ($P = 0.023$), *Chaetomium* ($P = 0.023$), and *Trichoderma* ($P = 0.004$). The associations between the occurrence of amoebae and the different groups of fungi and bacteria are presented in Fig. 1.

The amounts of amoebae and some fungi and bacteria were associated. The amounts of *Acremonium, A. versicolor, Chaetomium*, Sphaeropsidales and *Stachybotrys* on MEA medium, *Paecilomyces* on DG18 medium, and all bacteria were positively correlated with the amounts of amoebae, as presented in Table 2.

Differences between the building materials for the occurrence of amoebae were statistically significant (Mann–Whitney, $P = 0.002$). Among the various materials, amoebae seemed to favor wood (38% of the wood samples were positive for amoebae), but they only rarely occurred on mineral insulation (8%). However, the water content of the wood samples were remarkably higher than the water content of the mineral insulation samples (Kruskal–Wallis, $P = 0.000$).

Different types of ciliates, flagellates, and nematodes were also detected in the samples. The organisms found are presented in Table 3. The sizes of these organisms differed within flagellates between 3 and 6 μm and within ciliates from 20 to 40 μm. Of the 11 samples, flagellates and ciliates were found in all but 1. Also, nematodes were detected in a wood sample (wood 1) with severe damage.
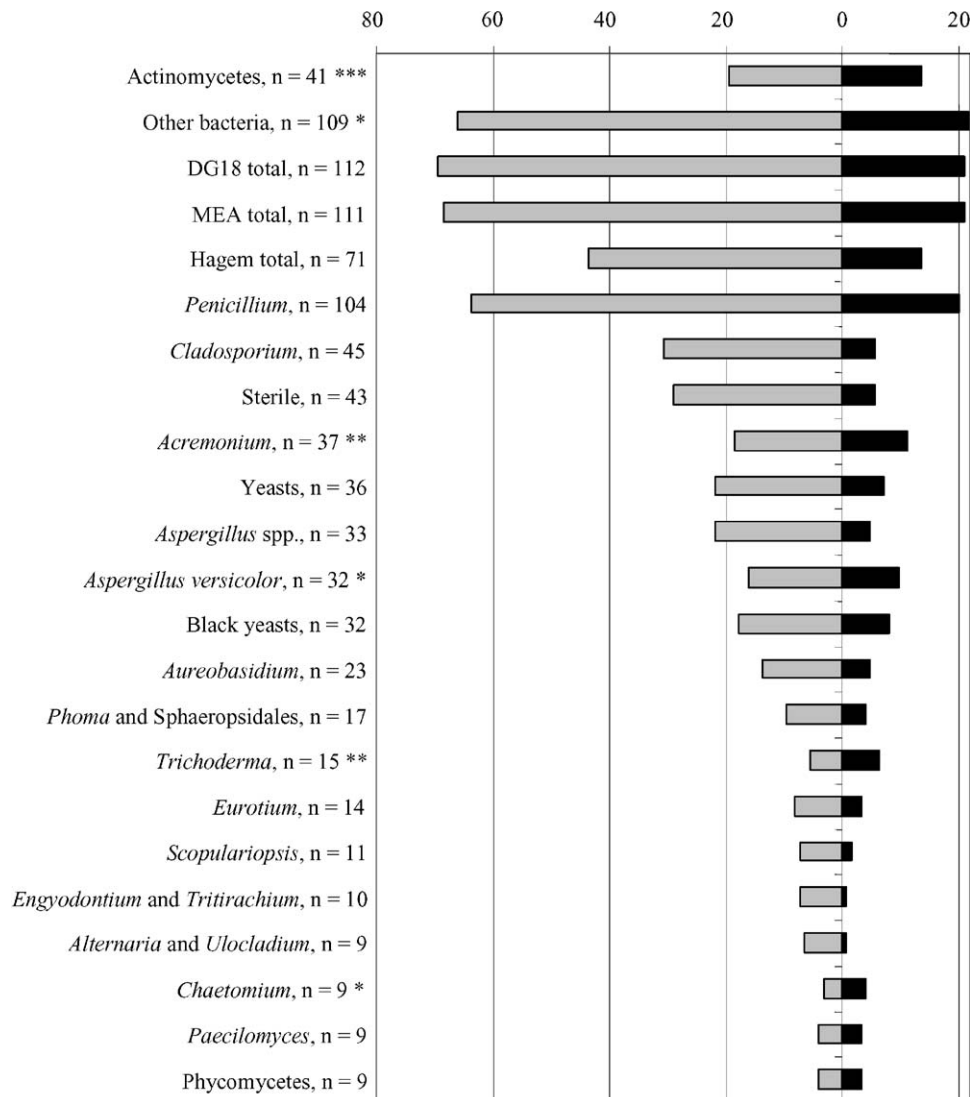
Fig. 1. The percentage of occurrence (of the total number of samples, $n = 124$) for each microbial genus or group without amoebae (□) and with amoebae (■). The maximum percentage of the stated microbial genus or group occurring with amoebae is 22%, as amoebae were found only in 22% of all samples.

4. Discussion

Many species of free-living amoebae are considered to be opportunistic or pathogenic organisms. In particular, species of *Acanthamoebae* and *Naegleriae* have been reported to cause often-fatal encephalitis, and *Acanthamoebae* are also known to be causative agents of grievous eye infections (Visvesvara, 1999). As no unexpected amoebiases have been reported among occupants of moisture-damaged buildings, a more important aspect of health effects worth considering with amoebae in damp buildings is the ability of amoebae to harbor bacteria that are possibly pathogenic to humans.

In this study, amoebae occurred together with several fungi that are regarded as indicator genera for moisture in buildings. We found that amoebae also favored cooccurrence with bacteria, especially actinomycetes. Actinomycetes and the fungi *A. versicolor*, *Stachybotrys* spp., and *Trichoderma* spp. are likely to occur when the water activity of the material is high ($a_w > 0.85$) (Samson et al., 1994). A possible explanation for the cooccurrence of amoebae and these microbes is similar moisture requirements. The organisms might also benefit from each other's presence, as amoebae feed on fungi and bacteria, among other small organic particles, and some bacteria are able to replicate inside of amoebae.

In addition to providing refuge to bacteria, amoebae have also been shown to enhance the virulence of some bacteria and their ability to enter and survive within macrophages (Cirillo et al., 1994, 1997, 1999). The resistant cyst form of amoebae provides a bactericide-free, nutrient-rich environment when conditions are too harsh for microbial growth (Inglis et al., 2000).

Table 2
Examples of association of amounts between amoebae with bacteria and certain fungi

		Amount of amoebae					Amount of amoebae		
		0	**1**	**2**			**0**	**1**	**2**
Actinomycetes	**0**	90[a]	12	6	*A. versicolor* on	**0**	95[a]	15	9
$p = 0.001^{**}$	**1**	7	5[a]	3[a]	MEA	**1**	2	1[a]	0
	2	0	0	1[a]	$p = 0.046^{*}$	**2**	0	1[a]	1[a]
Other bacteria	**0**	53[a]	2	1	*Chaetomium* on	**0**	96[a]	15	9
$p = 0.000^{***}$	**1**	32	10[a]	4[a]	MEA	**1**	1	2[a]	0
	2	12	5[a]	5[a]	$p = 0.036^{*}$	**2**	0	0	1[a]
DG18 total	**0**	45[a]	3	1	*Paecilomyces* on	**0**	97[a]	16	9
$p = 0.031^{*}$	**1**	39	10[a]	6[a]	DG18	**1**	0	1[a]	0
	2	13	4[a]	3[a]	$p = 0.036^{*}$	**2**	0	0	1[a]
MEA total	**0**	43[a]	2	1	Sphaeropsidales on	**0**	95[a]	14	9
$p = 0.005^{**}$	**1**	39	9[a]	4	MEA	**1**	1	1[a]	1[a]
	2	15	6[a]	5[a]	$p = 0.027^{*}$	**2**	1	2[a]	0
Acremonium on	**0**	89[a]	14	7	*Stachybotrys* on	**0**	97[a]	15	10[a]
MEA	**1**	2	2[a]	2[a]	MEA	**1**	0	1[a]	0
$p = 0.042^{*}$	**2**	6	1	1[a]	$P = 0.046^{*}$	**2**	0	1[a]	0

The amounts of microbes and amoebae are represented in three classes, of which 0 denotes little or none microbes or amoebae present, 1 denotes an intermediate amount of microbes or amoebae, and 2 means an abundant occurrence of microbes or amoebae.

[a] The number of samples within each group is shown. The number of samples in this group is greater than statistically expected number of samples.

Table 3
Protozoa found in the samples

Material	Water content (kg/kg)	Detected in the sample?		
		Flagellates	Ciliates	Amoebae
Wood 1	1.489	Yes	Yes	Yes
Wood 2	0.065	Yes	Yes	No
Gypsum board and cardboard	0.090	Yes	Yes	No
Mineral insulation	0.040	Yes	No	Yes
Concrete and paint 1	0.025	Yes	Yes	Yes
Concrete and paint 2	[a]	Yes	No	No
Plastic carpet	[a]	Yes	Yes	Yes
Chipboard 1	0.044	Yes	Yes	Yes
Chipboard 2	0.097	Yes	Yes	Yes
Chipboard 3	0.058	No	No	Yes
Chipboard 4	0.103	Yes	Yes	Yes

[a] The water content was not measured because the amount of the sample was too small.

Therefore, amoebae may have a notable effect not only on the spectrum of bacterial genera present in moisture-damaged buildings, but also on the harmfulness of these bacteria.

The present research indicates that differences in the occurrence of amoebae found between the building materials could also have been explained by the differences in the water contents of the materials. However, a stronger correlation has been shown between the water activity (relative moisture content, dependent on the characteristics of the material) of the material and the growth of microbes than between the water content and microbial growth (Pasanen et al., 2000). This phenomenon might also apply to amoebae. As the relationship between the water content and the water activity is different for different materials, the effect of water content on microbial growth also varies between materials. For example, the same water content for mineral insulation and pinewood results in remarkably lower water activity for the latter (Simonson et al., 2001). Therefore, the differences between the occurrence of amoebae in different materials are possibly explained not only with the water content of the samples, but also with the characteristics of the materials. The effect of the building material on the growth of amoebae needs to be studied further.

We found ciliates and flagellates in almost every building material sample analyzed. Based on these very preliminary results, ciliates and flagellates seem to be rather common in the severely moisture-damaged building materials, with no special distinction between the materials. Nematodes were found in only one sample; in that sample wood was very moist and badly damaged. As only 11 material samples were analyzed, however, conclusions should not be made about the influence of moisture, building material, or the occurrence of amoebae on these organisms. More research is needed to clarify the possible role of these protozoa as

exposing agents or of their ecological importance in the indoor environment.

In this study, the water content of the material greatly affected the occurrence and amounts of amoebae in the building materials. We found amoebae in all samples with high water content. This could be expected, since amoebae are usually found in water habitats or moist environments. The ability of some amoebae to form cysts, which are tolerant to desiccation, extremes of temperature, and a lack of nutrients, probably explains the findings of viable amoebae in dry samples (Rogerson and Patterson, 2000). Altogether, the present results indicate that amoebae are rather common in mold-damaged building materials and possibly may be a part of the complex microbial contamination associated with health symptoms in moisture-damaged buildings.

Acknowledgments

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