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Bacteria, Molds, and Toxins in Water-Damaged Building Materials

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Microbial toxins and eukaryotic cell toxicity from indoor building materials heavily colonized by fungi and bacteria were analyzed. The dominant colonizers at water-damaged sites of the building were *Stachybotrys chartarum* (10^3 to 10^5 visible conidia cm^{-2}), *Penicillium* and *Aspergillus* species (10^4 CFU mg^{-1}), gram-negative bacteria (10^4 CFU mg^{-1}), and mycobacteria (10^3 CFU mg^{-1}). The mycobacterial isolates were most similar to *M. komossense*, with 98% similarity of the complete 16S rDNA sequence. *Limulus* assay of water extracts prepared from a water-damaged gypsum liner revealed high contents of gram-negative endotoxin (17 ng mg^{-1} of *E. coli* lipopolysaccharide equivalents) and β-D-glucan (210 ng mg^{-1} of curdlan equivalents). High-performance liquid chromatography analysis of the methanol extracts showed that the water-damaged gypsum liner also contained satratoxin (17 ng mg^{-1}). This methanol-extracted substance was 200 times more toxic to rabbit skin and fetus feline lung cells than extract of gypsum liner sampled from a non-water-damaged site. The same extract contained toxin(s) that paralyzed the motility of boar spermatozoa at extremely low concentrations; the 50% effective concentration was 0.3 μg of dry solids per ml. This toxicity was not explainable by the amount of bacterial endotoxin, β-D-glucan, or satratoxin present in the same extract. The novel *in vitro* toxicity test that utilized boar spermatozoa as described in this article is convenient to perform and reproducible and was a useful tool for detecting toxins of microbial origin toward eukaryotic cells not detectable in building materials by the other methods.

Viable cells of microbes containing agents documented as hazardous in bioaerosols, such as gram-negative endotoxins (23) or β-1,3-glucan (6, 7) or mycotoxins (10), are seldom found in high airborne concentrations in houses in which moisture is a problem (moisture problem houses) (14). The shortcomings of methods available for sampling, isolation, and identification of microorganisms not forming colonies when injured and stressed by aerosolization were reviewed by Marthi (15). Studies concerning indoor air-related health hazards usually focus on one hazardous agent, measured by a single analytical technique. Identifying several emitting microbial contaminants directly within the building material is of interest when health hazards in a building are evaluated. Using a multimethod approach, we demonstrated in this study that water-damaged indoor building materials contained substances of microbial origin extremely toxic to eukaryotic cells and large quantities of several microbial species known to be potentially toxic or immunopotentating, among which were new species and species not earlier reported in connection to water-damaged indoor building materials.

MATERIALS AND METHODS

Sampling of building materials. The building studied, a children's day care center, flat roofed and built in 1974, had a history of repeated water damage since 1980 and was classified as a moisture problem house by the local health authority. The roof was renovated in 1990. According to a survey, 5 of 19 workers reported irritation of nose and eyes as a prevalent symptom associated with working conditions and indoor air, while 40% complained of smell and 35% of stuffness. Indoor building materials and settled dusts were sampled from children's two residence rooms exhibiting visible water damage. Sampling was done late in 1995 during renovation work, when the indoor walls and ceilings were demolished, exposing gypsum liners with signs of old dehydrated water damage. Visibly water-damaged sites and sites with no visible water damage were also sampled.

Microscopic analyses. The building materials sampled from the water-damaged sites and non-water-damaged sites were analyzed by light microscope, scanning electron microscope (SEM), and transmission electron microscope (TEM) by protocols described elsewhere (2). The amounts of fungal propagules were estimated by the tape sampling method.

Media and chemicals. The microbiological media and diluent for serial plating were from Difco (Detroit, Mich.) unless otherwise stated. The following chemicals were obtained from Sigma (St. Louis, Mo.): T2 toxin, verrucarin A, ochratoxin, roridine, and cycloheximide. Reference satratoxins (G and H) were a gift from Kirsti Liukkonen. Reference endotoxin, *Escherichia coli* B6 lipopolysaccharide (LPS) was from Biowhittaker (Walkersville, Md.), and reference β-D-glucan (curdlan) was from Wako Pure Chemical (Japan). Other chemicals were from the local suppliers and were of analytical quality.

Microbiological analyses. Prior to cultivation the bacteria of the dehydrated building materials were resuscitated, viable cells were counted, and bacterial strains were isolated and identified as described elsewhere (2, 9, 27). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of PCR products were carried out as described previously (22). Purified PCR products were sequenced with the Taq Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.). Sequence reaction mixtures were electrophoresed with the Applied Biosystems 373A DNA Sequencer. The 16S rDNA sequence was manually aligned against representative sequences of members of the domain bacteria. The sequence similarity to the nearest relative was determined as an indication of identity.

For isolation of different fungal species from gypsum liner dichloran agar with 18% glycerol (DG18), malt extract agar (MEA) and MEA with added 20 g of glucose per liter, Czapek Yeast Extract agar, and glycerol nitrate agar (G25N) were used as according to Verhoeff et al. (32). For isolation of the slowly growing organisms CMA medium (corn meal agar) was used (26). The isolates were identified according to Domsch et al. (4), Gravesen et al. (8), Pitt (20), and Samson et al. (26). The total fungal plate counts were determined on CMA, after incubation at 20 to 23°C for 14 days.

For the assay of gram-negative bacterial endotoxin and β-D-glucan, the indoor

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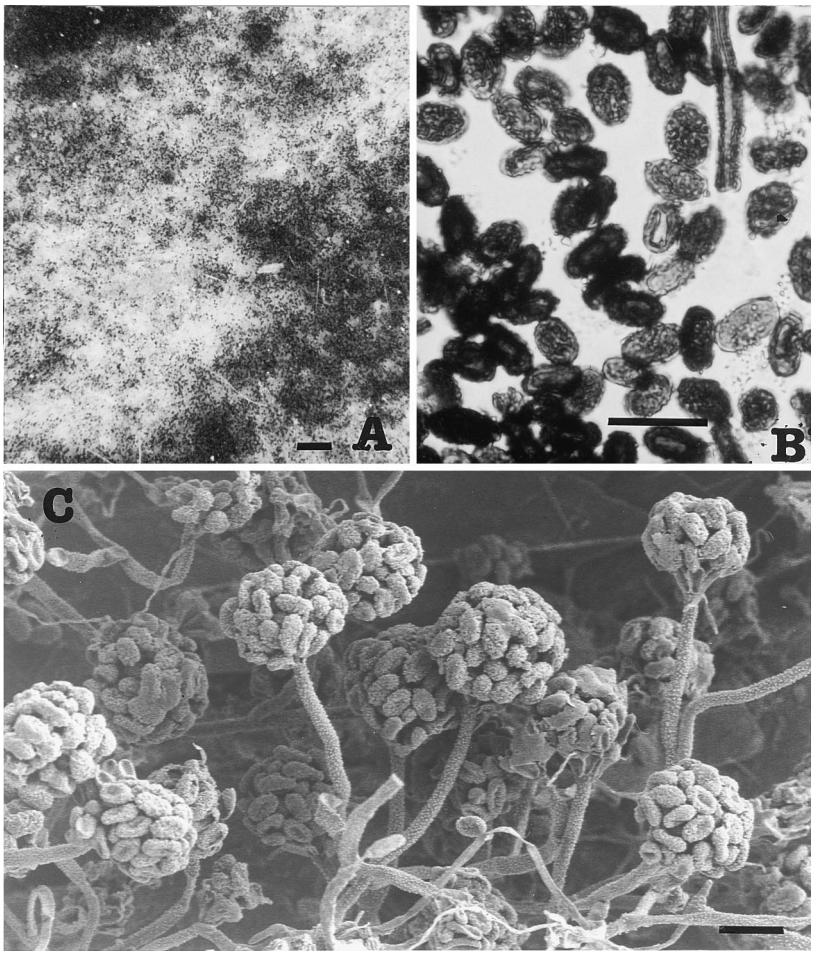

FIG. 1. Microscopic views of water-damaged gypsum liner (site 1, Table 1). (A) Stereo microscopic view. The surface of the gypsum board liner was discolored by unevenly distributed black spots. Bar, 500 μm. (B) Surface of the gypsum board liner sampled onto Scotch tape, photographed by bright field microscopy, revealing a homogenous and dense population of conidia similar to the species of *Stachybotrys*. Bar, 10 μm. (C) Scanning electron micrograph of the same site as in panels A and B. It shows the liner surface covered with dense mycelia and conidiophores with wartlike ornamentation characteristic of the microfungus *Stachybotrys*. Bar, 10 μm.

building materials were water extracted and the extracts were tested for *Limulus* activity by LAL assay, using the standard kinetic turbidimetric LAL assay performed by the SCAN DIA Laboratory Service (Copenhagen, Denmark). *Limulus* activities in bacterial pure cultures were assayed by using the gel clotting modification of the *Limulus* test calibrated with *E. coli* B6 LPS (Pyrogent; Biowhittaker).

For eukaryotic toxicity assays, water-damaged gypsum board liner visibly contaminated by fungi (0.4 g) and non-water-damaged control gypsum board liner (0.4 g) with no visible fungal contamination were extracted with 95% methanol overnight. Filtered extracts were vacuum evaporated, and the residues were dissolved in a known volume of 100% methanol and divided into four aliquots: three for biological toxicity tests and one for chemical analysis. Methanol was evaporated under an atmosphere of nitrogen.

Toxicity testing. For cytotoxicity testing with a continuous feline fetus lung (FFL) cell line, the evaporated residue was dissolved in 10% methanol in phosphate-buffered saline (0.1 M), pH 7.2, to a concentration that corresponded to 1 mg of evaporated solids per ml. Fifty percent inhibition of the cell proliferation was evaluated with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] bioassay (3).

Toxicity to boar spermatozoa was tested with dimethyl sulfoxide (DMSO) solution of the evaporated solids obtained by methanol extraction of the building material (8 mg of solids ml^{-1}). The test was carried out as follows: boar ejaculate was filtered through a gauze to get rid of the gel. The semen was diluted to 18% with a commercial semen extender, MR-A (Kubus, S.A., Madrid, Spain), to a density of 40 to 70 $\times$ 10^6 spermatozoa ml^{-1} (24). The MR-A-diluted semen was gently mixed and administered into plastic screw-capped test tubes (2 ml per tube). The tubes were pretested and used only if found to be nontoxic to boar spermatozoa. The building material extract was diluted by serial twofold dilutions in DMSO from 8 mg to 30 μg (dry weight) ml^{-1}. A volume of 20 μl of each dilution was administered to 2 ml of MR-A-diluted boar semen. The test tubes were incubated at room temperature and turned upside down once a day. Motility of the exposed spermatozoa was measured after 3, 4, and 5 days by assessment of rapid motility of sperm cells by using a computerized motility analyzer (HTM-S, ver. 7.2; Hamilton-Thorn Research, Danvers, Mass.) as described by Andersson and Katila (1) and confirmed by visual assessment of sperm motility (11). M-RA-diluted spermatozoa (2 ml) exposed to 20 μl of DMSO were used as a blank assay. The test was recorded positive when both the instrumental and the visual methods assessed a ≥50% decrease in sperm motility of the exposed spermatozoa compared to the control spermatozoa. The morphology of the mitochondria of the spermatozoa were examined by TEM according to Rabkin et al. (21). For rabbit skin test the methanolic extract was evaporated and dissolved in acetone to a concentration of 8 mg of evaporated residue per 100 μl, and the test was performed according to the method of Pasanen et al. (18).

Assay of satratoxin. Satratoxins (G and H) were analyzed from the methanolic extracts of building materials by high-performance liquid chromatography (HPLC). The retention times were compared with those of reference satratoxin (G and H, a mixture). To confirm the identity of macrocyclic trichothecenes, the extracts were hydrolyzed for observation of verrucarol (19).

Nucleotide sequence accession number. The EMBL accession number for the *Mycobacterium* strain MA-112/96 is YO8857.

RESULTS

Microscopic inspection. Gypsum liners from interior walls and ceilings were examined by light microscopy and SEM and TEM. Figure 1A and B shows light-microscopic views of a gypsum board liner from a water-damaged site. As seen in the figure, the gypsum liner paper surface was heavily contami-

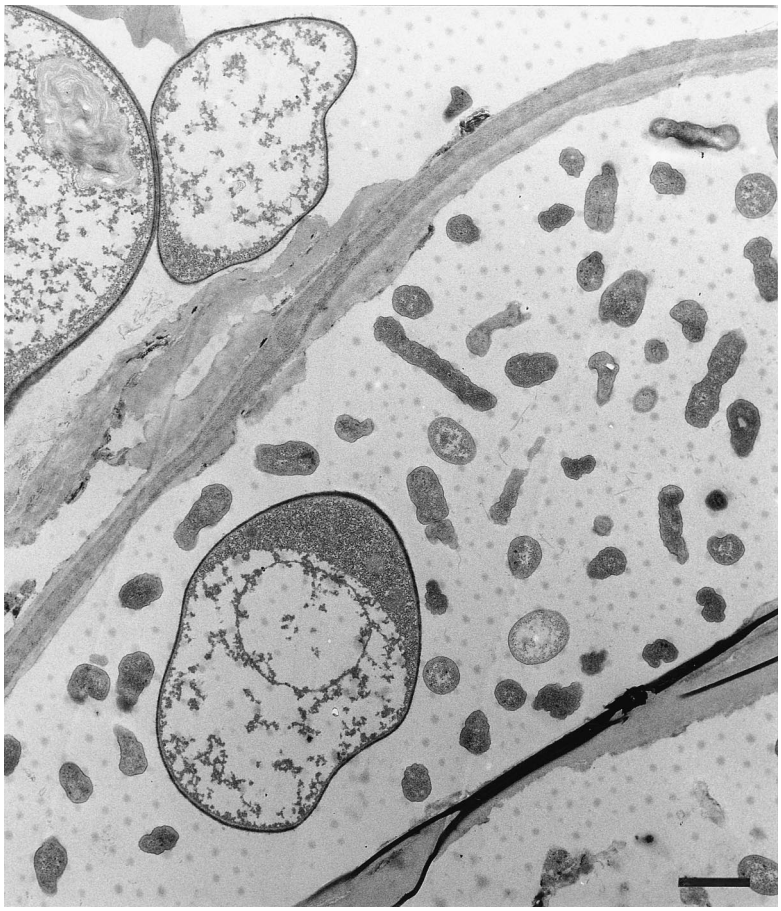

FIG. 2. Transmission electron micrograph of the water-damaged gypsum liner (site 1). Cross sections of the liner containing fungal hyphae and bacteria are shown.

nated by fungi. The same liner paper examined by SEM is shown in Fig. 1C. Figure 1C shows that the morphologies of hyphae, conidiophores, and conidia colonizing the gypsum board liner resembled a monoculture of a *Stachybotrys* sp., but thin sections (TEM) showed in addition colonization by a diverse bacterial community (Fig. 2).

Eleven different sites composed of indoor building materials (including four samples of gypsum liners) were inspected, and the visible microbes were enumerated by light microscopy. The results revealed that samples from 9 of 11 sites with a history of water-damage contained conidia recognized a *Stachybotrys* sp. as the dominant organism. The microscopic count of conidia ranged from 10^3 to 10^5 conidia per cm^2. No fungal contamination (less than 10^2 propagules per cm^2) was detected in the materials collected from sites other than those with a history of water damage.

Microbial biomarkers, endotoxin, β-D-glucan, and satratoxin in the indoor building materials of the day care center. Seven samples of indoor building materials were analyzed for endotoxin-specific *Limulus* activity indicative for gram-negative bacteria and for β-D-glucan-specific *Limulus* activity indicative of the presence of fungal biomass. The results in Table 1 indicated high endotoxin-specific (17 μg of *E. coli* LPS g^{-1}) and β-glucan-specific (210 μg of β-D-glucan g^{-1}) *Limulus* activities in water-damaged gypsum liner. The β-glucan-specific *Limulus* activity of the water-damaged gypsum liner (site 1) was almost two times higher than that of a pure culture of *Paecilomyces variotii* 95/111 (127 μg g^{-1}) used as reference material. *Limulus* activities were 10 to 100 times higher in materials collected from areas exhibiting signs of water damage (sites 1, 3, and 5) than in materials collected from areas obviously not damaged by water (site 13).

Gypsum liners from a water-damaged site, heavily contaminated with *Stachybotrys* spp. (site 1) and from a nondamaged area, not visibly contaminated by microbes (site 13), were analyzed for satratoxin. Satratoxins G and H were found (17 μg g^{-1}) in the visibly contaminated area of the gypsum board liner (site 1) but not in a nondamaged area of the same gypsum board liner ($\leq$1 μg g^{-1}, site 13). Verrucarol was detected from fungus-contaminated gypsum board liner extract after hydrolysis, confirming the identity of the satratoxins G and H. No satratoxin or verrucarol ($<$1 μg g^{-1}) was detected from the same gypsum board liner taken from a non-water-damaged area. In conclusion, high contents of endotoxin, β-D-glucan, and satratoxin occurred in the same location as the microscopically observed heavy colonization by *Stachybotrys* spp. in the water-damaged gypsum board liner, while none was found in the non-water-damaged areas.

Quantitation of culturable bacteria and fungi in the building materials. The bacterial colonization of indoor building materials was investigated by colony counting the culturable cells (Table 2). Gypsum board liner from the water-damaged areas contained 10^3 psychrotrophic, 10^4 mesophilic, and 10^2 thermotrophic bacteria mg^{-1}, which is over 100 times more than was found in samples taken from the non-water-damaged areas of the same gypsum board liner (Table 2).

TABLE 1. Endotoxin, β-D-glucan, and satratoxin in the indoor building materials of a children's day care center and reference materials

Sampled material	Site no.	Amt (μg g^{-1}) of toxins[a]		
		Endotoxin[b]	β-D-glucan[c]	Satratoxin[d]
Day care center				
Water-damaged gypsum board liner	1	17	210	17
Water-damaged gypsum board liner	3	2.3	14	
Water-damaged mineral wool	5	8	2.5	
Ceiling coating paper (randomly chosen)	6	4.2	41	
Settled dust from floor collected during demolishing	10	0.17	0.5	
Mineral wool (randomly chosen)	11	0.1	<0.1	
Gypsum board liner (not water damaged)	13	0.41	0.4	< 1
Reference materials				
Pure culture (dry) of fungus (*P. variotii* 95/111)		0.01	127	
Hay dust sampled from cow shed		27	1.9	

[a] The average difference between duplicate samples was ±5%.
[b] Endotoxin was measured as *Limulus* activity expressed as micrograms of *E. coli* B LPS equivalents.
[c] β-D-glucan was measured as *Limulus* activity and expressed as micrograms of curdlan (β-1,3-glucan) equivalents.
[d] Satratoxin was measured by HPLC with purified satratoxin (G and H) as a standard.

TABLE 3. Bacterial genera isolated from gypsum board liners of children's day care center interior walls

Isolate(s)	Water-damaged area		Nondamaged area	
	No. of strains isolated	Density in board liner (CFU mg^{-1})	No. of strains isolated	Density in board liner (CFU mg^{-1})
Gram-positive mesophiles				
Arthrobacter sp.	5	10^3	1	1
Bacillus (*B. pumilus*)	4	10^3	0	<1
Cellulomonas sp.			2	1
Gordona sp.	1	10^2	0	<1
Micrococcus sp.	3	10^3	3	10
Mycobacterium sp.	6	10^3	0	<1
Paenibacillus (*P. macerans* and *P. polymyxa*)	5	5×10^2	0	<1
Spore-forming actinomycetes				
Streptomyces sp.	5	10^2	5	20
Other	1	10^2	0	<1
Gram-negative mesophiles (endotoxin positive)[a]				
Agrobacterium sp.	10	3×10^3	0	<1
Caulobacter sp.	4	3×10^3	0	<1
Stenotrophomonas sp.	1	10^2	0	<1
Chryseomonas luteola	0	<1	1	1
Thermotrophic bacteria				
B. amyloliquefaciens	2	10^2	0	<1
Cumulative total	47	10^4	12	33

[a] As judged from positive *Limulus* assay reaction.

Total fungal plate count obtained on CMA agar from water-damaged gypsum liner (site 1 in Table 1) was 8,000 CFU mg^{-1}. About 1,500 CFU mg^{-1} of these represented *Stachybotrys chartarum*. Fungi culturable on MEA medium were isolated from the gypsum liners, board liners, paper, and mineral wool sampled from the day care center. Sixteen taxa of molds were detected from different building materials and identified to genus or species level. *Penicillium aurantiogriseum* Dierckx., *P. citrinum* Thom, and other *Penicillium* species were the most frequently isolated taxa. Other taxa identified to species level were *Aspergillus versicolor* (Vuill.) Tiraboshi, *Chaetomium globosum* Steud., *Cladosporium herbarum* (Pers.: Fr.) Link, *C. sphaerospermum* Penz., *Mucor hiemalis* Wehmer, *M. plumbeus* Bonord., and *Paecilomyces variotti* Bain. In spite of the fact that the only fungus detectable by microscopic examination was morphologically recognizable as a *Stachybotrys* spp., this fungus was not cultivable from the water-damaged gypsum liner on MEA medium but could be enumerated on CMA.

Isolation and identification of bacteria. Fifty-nine aerobic bacterial strains were isolated at 50, 22, and 16°C from gypsum board liners sampled from water-damaged and nondamaged areas and identified to genus level. The results, shown in Table 3, indicate that the diversity of the bacteria in water-damaged gypsum liners was larger and different from that in nondamaged gypsum liners. The following bacterial taxa were found in the water-damaged area but not in the nondamaged area: *Bacillus amyloliquefaciens* (thermotrophic), *B. pumilus*, *Paenibacillus polymyxa*, and strains of *Caulobacter*, *Agrobacterium*, and *Mycobacterium* spp. The results in Fig. 2 and Table 3 indicate that the water-damaged area of the gypsum board liner was inhabited by a rich and diverse bacterial flora possibly specific to this habitat, dominated by endotoxin-containing gram-negative bacteria and *Mycobacterium* species. Mesophilic spore-forming actinomycetes and *Micrococcus* spp. were found in both areas. The identities of the mycobacterial strains (6 strains), *Agrobacterium* strains (10 strains), and *Caulobacter*

TABLE 2. Colony counts of bacteria cultivable from gypsum liners of interior walls in children's day care center

Material tested	Sampling site	Bacterial viable count (10^3 CFU mg^{-1})[a] at the following growth temperature (°C):		
		16	22	50
Water-damaged gypsum liner ($n = 2$)	1	3.2 (±37%)	10 (±42%)	0.10 (±50%)
Gypsum liner (not water damaged) ($n = 2$)	13	0.09 (±40%)	0.04 (±42%)	<0.001

*CFU were counted on tryptic soy agar plates after 7 and 14 days of incubation. Data in parentheses are average differences between replicate samples.

TABLE 4. Toxicity of methanol-extractable dry solids from gypsum board liners and settled dust from children's day care center

Site of sampling	Material tested	Source of culturable microbial contamination[a]	EC_{50}[b] (μg [dry wt] ml^{-1})[b]	
			FFL cells	Boar spermatozoa
1	Water-damaged gypsum board liner	*Stachybotrys* sp., *Penicillium* sp., bacteria	1.5 (0.9)	0.29 (0.19)
10	Settled dust	Bacteria, *Penicillium* (*Stachybotrys*) sp.	1,280	25
13	Gypsum board liner (not water damaged)	Bacteria	270 (250)	54 (50)

[a] For description of microbial quality of the tested materials, see Tables 1, 2, and 3.
[b] Expressed as the endpoint dilution of methanol-extracted material (dry weight) causing <50% inhibition of cell proliferation or 50% decrease in sperm motility. The average difference between duplicate samples was ±6% when toxicity toward FFL cells was tested and ±10% when independently sampled ejaculates of boar spermatozoa were tested for toxicity in duplicate samples. Data in parentheses are equivalent to 1 mm^2 of gypsum liner.

strains (4 strains) were confirmed by a battery of physiological tests and sequencing of 16S rDNA. The full 16S rDNA sequence was analyzed for one of the mycobacterial strains, strain MA-112/96, and revealed <98% similarity to any described mycobacterial species, the closest being *Mycobacterium komossense* (type strain). Biochemical characterization showed that these mycobacterial strains were rapidly growing mycobacteria, very likely a new species, which will be described in detail elsewhere.

Toxic substances extractable from the building materials. Compounds extractable into methanol from the gypsum liners and settled dust were assayed for toxicity toward FFL cells (cytotoxicity) and boar spermatozoa (sperm toxicity) and by rabbit skin test (skin toxicity). The results in Table 4 demonstrate that the toxicity thresholds (50% effective concentration [EC_{50}]) toward FFL cells were 270 μg of extracted solids ml^{-1} for nondamaged gypsum board liner and 1.5 μg ml^{-1} for gypsum board liner visibly contaminated by fungi. The skin toxicity threshold was 7,600 μg of methanol extractable solids ml^{-1} from gypsum board liner visibly contaminated by fungi. No toxic reactions were obtained for the nondamaged gypsum board liner (detection limit, 1,000 μg ml^{-1}). Toxicity toward boar spermatozoa was repeated with two separate semen ejaculates four times. The toxicity threshold (EC_{50}) toward boar spermatozoa was 54 μg ml^{-1} for the nondamaged gypsum board liner extract and as low as 0.29 μg ml^{-1} for extract of the gypsum board liner visibly contaminated by fungi.

The yield of methanol-extractable substances from 1 g of contaminated gypsum board liner was 9.5 mg (dry weight). This is 2.75 mg (dry weight) more than the yield from 1 g of nondamaged gypsum board liner. This excess of 2.75 mg g^{-1} may thus contain substances of microbial origin and was possibly responsible for the toxicity of the methanol extract made from gypsum board liner from a water-damaged area being 180-fold higher than that of the substances extractable from the gypsum board liner taken from an area with no visible water damage (Table 4).

The boar spermatozoa and the FFL cells were tested for toxicity of several mycotoxins, satratoxin (G and H), T-2 toxin, verrucarin A, ochratoxin, and roridine (Table 5). The boar spermatozoa were relatively insensitive to satratoxin and the other mycotoxins, and the toxicity thresholds, EC_{50}, were over 500 times greater than those for the FFL cells (Table 5). The boar spermatozoa were also relatively insensitive to endotoxin and β-D-glucan; EC_{50} was >1 μg ml^{-1}. If it is assumed that the excess of methanol-extractable matter of microbial origin was solely responsible for the toxicity toward boar spermatozoa, its calculated toxicity threshold was 84 ng (dry weight) of methanol-extractable dry solids per ml of sperm suspension. This 84 ng then also contained 0.42 ng of endotoxin (as *E. coli* LPS equivalents), 5 ng of β-1,3-glucan (curdlan equivalents), and 0.5 ng of satratoxin (G and H). The latter amounts found in the extract from water-damaged gypsum liners, 0.5 ng ml^{-1}, thus did not explain the observed toxicity toward boar spermatozoa. FFL cells were very sensitive to satratoxin; EC_{50} was 0.9 ng ml^{-1}. Toxicity of the methanol-extracted solids of the gypsum board liner observed toward FFL cells was explained by satratoxin, but toxicity observed toward boar sperm motility (Table 4) must have been caused by a different toxin.

Morphological changes in spermatozoa in response to exposure to the substances from gypsum board liners are shown in Fig. 3. Figure 3A shows a cross section of the mitochondrial sheath of the spermatozoan tail after exposure to methanol extract from non-water-damaged gypsum board liner. The morphology was identical to that of nonexposed, healthy spermatozoa (not included in the figure). Figure 3B shows a cross section of spermatozoa after exposure to extract from water-damaged gypsum board liner. The figure displays a typical example of spermatozoa with malformed mitochondria found in most cells exposed to methanol-extracted solids from gypsum board liner taken from a water-damaged area. Mitochondria of the affected sperm cells were swollen and partially disrupted, possibly explaining the inhibited sperm motility. The frequence of mitochondrial swelling was related to the exposing dose of the methanol extract of the water-damaged gypsum board liner. We therefore conclude that the agent causing the mitochondrial damage in the spermatozoa must have originated from the water damage to the gypsum board liner and very likely was of microbial origin. Boar spermatozoa thus proved to be useful for *in vitro* toxicity testing of compounds affecting mitochondrial membranes and ATP synthesis.

DISCUSSION

We report on the microbial colonization and the presence of selected potentially hazardous microbial agents in construction materials of the interior walls in a moisture problem house. Water-damaged building materials hidden inside the building

TABLE 5. Testing of FFL and boar spermatozoa against reference toxins

Reference toxin	EC_{50} (μg [dry wt] ml^{-1})	
	FFL	Boar spermatozoa
Satratoxin (G and H)	0.0009	5
T-2 toxin	0.0023	0.5
Verrucarin A	0.0006	5
Ochratoxin A	ND[a]	>10
Roridin A	0.0007	>1
Endotoxin	ND	>1
β-D-Glucan	ND	>1
Cycloheximide	ND	>4

[a] ND, not determined.

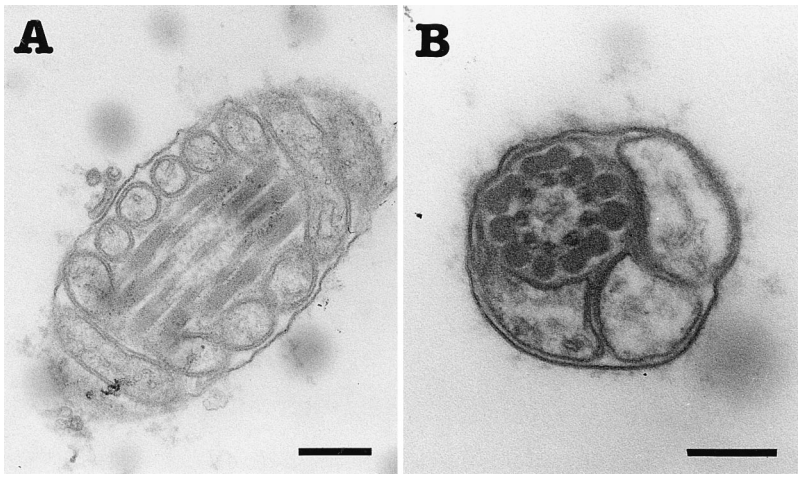

FIG. 3. Thin sections through the midpiece of boar spermatozoa displaying the effect of methanol-extracted substances from gypsum board liners on the structure of the mitochondrial sheath. The sperm cells were exposed to methanol-extractable solids from a nondamaged gypsum board liner (site 13, Table 1) (A) or a water-damaged site of gypsum liner (site 1, Table 1) (B). The boar spermatozoa shown were exposed to 2 μg of the solids (dissolved in DMSO) per ml of sperm cell suspension. (A) Mitochondria of ordinary size with intact membranes; (B) swollen mitochondria with demolished membranes. The frequence of swollen mitocondria was 68% (±5%) after exposure to 1 μg ml^{-1} and 81% (±3%) after exposure to 2 μg of methanol-extracted substances from water-damaged gypsum liner ml^{-1}. After exposure to 2 μg of methanol-extracted substances from non-water-damaged areas of gypsum liner ml^{-1}, the frequency of the swollen mitocondria was <1%. Bar, 200 nm.

constructions and colonized by microorganisms have been suspected as a source of bioaerosol emission in so-called mold problem houses (8, 12, 28).

Immunogenic, physiologically reactive, and toxic cell wall components or products of microorganisms, documented as hazardous agents expected in bioaerosols, include peptidoglycans, gram-negative endotoxin, β-1,3-D glucans, lipoglycans, and teichoic acids and mycotoxins such as satratoxins (16, 23, 29, 33). Mycobacteria, very likely representing a new species, were found in large quantities in water-damaged gypsum liners (10^6 CFU g^{-1}). Cell wall components (the muramyl peptides, arabinogalactan, and the mycolic acids) of the genus *Mycobacterium* are known to be toxic and to be strong modulators of the immune system, e.g., release of cytokines and pyrogenic and somnogenic activity (5, 13). Microbial components and products are also known to amplify the hazardous effects of one another and alter immune functions (6, 13, 30, 31).

Strains of the genus *Stachybotrys* are known to produce many bioactive and/or toxic compounds (17, 25). Trichothecene mycotoxins known to be included in the conidia of *S. chartarum* (28) may affect cellular membranes, inhibit synthesis of protein and nucleic acids, and induce lymphocyte depletion and necrosis in both the B-cell- and T-cell-dependent regions of the lymphoid organs (12). In this study *S. chartarum* and also other fungi typical of moisture problem houses were isolated from the building materials, e.g., *A. versicolor* and *P. aurantiogriseum*. Strains of the latter two fungi were previously described to produce hepatotoxic sterigmatocystin and nephrotoxic ochratoxin A in foods and animal fodder (26). Experimental growth of *A. versicolor* has been shown to produce sterigmatocystin on wall paper-glue agar (8), but the toxin production abilities of these fungi in indoor environments are not known.

Highly toxic *S. chartarum* propagules of the gypsum liner escaped culturing by routine methods for cultivation described here, although they were microscopically visible and dominant on surfaces of liners taken from the water-damaged area of indoor walls and were also indicated for by the presence of chemically assayed satratoxin in the liner. The germination and growth of *S. chartarum* on MEA medium is slow compared to those of many other genera. Only a small percentage of the *Stachybotrys* conidia are usually viable in laboratory cultures, and their growth is inhibited by several species of *Penicillium* (8). Plating on CMA medium seemed to favor slowly growing fungi like *Stachybotrys* species, which are able to utilize a poor growth medium. Dominance of the slowly growing *S. chartarum* in a mold population growing on the interior building materials may indicate the presence of an old and stable microbial community. The coincidence of large amounts of the cellulolytic (8) fungus *Stachybotrys* with potential dinitrogen fixers (*Agrobacterium* and *Paenibacillus* species) in the water-damaged gypsum liner may imply a synergistic relationship between these organisms, possibly explaining their massive proliferation in a poor growth environment.

The results of the assay for gram-negative endotoxin and the counting of viable cells on the water-damaged gypsum liner supported each other. *Limulus* assay revealed a high content of gram-negative bacterial endotoxin and confirmed by viable counting large amounts of gram-negative endotoxin-containing species, e.g., *Caulobacter* and *Agrobacterium* spp. Observed large amounts of β-D-glucan coincided with microscopically observed fungal colonization of the indoor building materials.

The three independent methods for toxicity assay used in this study gave results that seemed confusing at first sight. The cytotoxic and skin toxic effects noticed could be explained by the satratoxin, but the extreme toxicity of the methanol-extractable solids from water-damaged gypsum board liners toward boar spermatozoa indicated the presence of yet unknown toxins. The electron microscopically observed swelling and disruption of mitochondria as well as the extincted motility of the sperm cells indicate interference by the microbial toxins with mitochondrial functions via yet unknown mechanisms. The boar spermatozoa in vitro toxicity test described in this study is an attractive tool for monitoring toxicity toward eukaryotic cells, as it involves no damage or pain to the animal and is convenient to perform and reproducible also when independent ejaculates were intercompared.

When the microbiological and toxicity assays have been summarized, our results show that the water-damaged gypsum liner covering the indoor walls and ceiling of a children's day care center not only was heavily contaminated by microbes but

also contained a high load of several toxic and immunoreactive agents. In addition it contained components that paralyzed sperm motility at extremely low doses. Our results indicate that water-damaged indoor building materials may emit bioreactive agents, and we assume that several of them may be involved in the etiology of health symptoms associated with indoor air. Several analytical techniques used in parallel are needed when building-related health hazards are investigated.

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REFERENCES

1. **Andersson, M., and T. Katila.** 1992. Evaluation of frozen-thawed stallion semen with a motility analyzer, p. 1837–1839. *In* The Hague Congress proceedings 4. The 12th International Congress in Animal Reproduction, 23 to 27 August 1992. The Hague, Netherlands.
2. **Andersson, M., M. Laukkanen, E.-L. Nurmiaho-Lassila, F. Rainey, S. Niemelä, and M. Salkinoja-Salonen.** 1995. *Bacillus thermosphaericus sp. nov.* a new thermophilic ureolytic *Bacillus* isolated from air. Syst. Appl. Microbiol. **18:**203–220.
3. **Dombrink-Kurtzman, M. A., G. Bennet, and J. Richard.** 1994. An optimized MTT bioassay for determination of cytotoxicity of fumonicins in turkey lymphocytes. J. Assoc. Off. Agric. Chem. **77:**512–516.
4. **Domsch, K., W. Gams, and T. H. Anderson.** 1980. Compendium of soil fungi, vol. 1. Academic Press, London, U.K.
5. **Draper, P.** 1982. The anatomy of the mycobacteria, p. 36–37. *In* C. Ratledge and J. Stanford (ed.), The biology of mycobacteria. Academic Press, London, U.K.
6. **Fogelmark, B., M. Sjöstrand, and R. Rylander.** 1994. Pulmonary inflammation induced by repeated inhalation of (1-3) β-D-glucan and endotoxin. Int. J. Exp. Pathol. **75:**85–90.
7. **Fox, A., and R. Rosario.** 1993. Quantitation of muramic acid, a marker for bacteria, in dust from hospital and home air conditioners using gas chromatography-mass spectrometry, p. 267–272. *In* Indoor Air—93. Proceedings of the 6th International Conference on Indoor Air Quality and Climate, vol. I. Helsinki, Finland.
8. **Gravesen, S., J. C. Frisvad, and R. A. Samson.** 1994. Microfungi, p. 49–50. Munksgaard Publishers, Copenhagen, Denmark.
9. **Greiner-May, E., R. Kroppenstedt, E. Korn-Wendish, and H. Kutzner.** 1987. Morphological and biochemical characterisation and emended description of thermophilic actinomycetes species. Syst. Appl. Microbiol. **61:**97–109.
10. **Hendry, K., and E. Cole.** 1993. A review of mycotoxins in indoor air. J. Toxicol. Environ. Health **38:**183–189.
11. **Januskauskas, A., and H. Rodriguez-Martinez.** 1995. Assessment of sperm viability by measurement of ATP, membrane integrity and motility in frozen/thawed bull semen. Acta Vet. Scand. **36:**571–574.
12. **Johanning, E., R. Biagini, D. Hull, P. Morey, B. Jarvis, and P. Landsbergis.** 1996. Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment. Int. Arch. Occup. Environ. Health **68:**207–218.
13. **Johnson, A. G.** 1994. Molecular adjuvants and immunomodulators: new approach to immunization. Clin. Microbiol. Rev. **7:**277–289.
14. **Maroni, M., B. Seifert, and T. Lindvall (ed.).** 1995. Indoor air quality. Air quality monographs, vol. 3, p. 149–160. Elsevier Science B. V., Amsterdam, The Netherlands.
15. **Marthi, B.** 1994. Resuscitation of microbial aerosols, p. 192–225. *In* B. Lighthart and A. Moore (ed.), Atmosphaeric microbial aerosols. Chapman & Hall, New York, N.Y.
16. **Miller, J. D.** 1994. Mycotoxins, p. 87–94. *In* R. Rylander and R. Jacobs (ed.), Organic dust, exposure, effects and prevention. Lewis Publishers, London, U.K.
17. **Nikulin, M., A.-L. Pasanen, S. Berg, and E.-L. Hintikka.** 1994. *Stachybotrys atra* growth and toxin production in some building materials and fodder under different relative humidities. Appl. Environ. Microbiol. **60:**3421–3424.
18. **Pasanen, A.-L., M. Nikulin, M. Tuomainen, S. Bergs, P. Parikka, and E.-L. Hintikka.** 1993. Laboratory experiments on membrane filter sampling of airborne mycotoxins produced by *Stachybotrys atra*. Atmosph. Environ. **27:** 9–13.
19. **Pasanen, A.-L., M. Nikulin, S. Berg, and E.-L. Hintikka.** 1994. *Stachybotrys atra* (*corda*) may produce mycotoxins in respiratory filters in humid environment. Am. Ind. Hyg. Assoc. J. **55:**62–65.
20. **Pitt, J. I.** 1991. A laboratory guide to common *Penicillium* species, 2nd ed. Commonwealth Scientific and Industrial Research Organization, North Ryde, New South Wales, Australia.
21. **Rabkin, S., J. Friesen, J. Ferris, and Y. Fung.** 1979. A model of cardiac arrhythmias and sudden death: cantharidin-induced toxic cardiomyopathy. J. Pharmacol. Exp. Ther. **210:**4350–4355.
22. **Rainey, F. A., M. Dorsch, H. W. Morgan, and E. Stackebrandt.** 1992. 16S rDNA analysis of *Spirochaeta thermophila*: position and implications for the systematics of the order *Spirochaetales*. Syst. Appl. Microbiol. **16:**224–226.
23. **Rylander, R.** 1995. Endotoxins in the environment, p. 79–90. *In* J. Levin, C. R. Alving, R. S. Munford, and H. Redl (ed.), Bacterial endotoxins. Progress in clinical and biological research, vol. 392. John Wiley and Sons, New York, N.Y.
24. **Rättö, J., and L. Jokinen.** 1990. Reports about number of swine inseminations and farrowing results in Finland 1989, comparison between two diluents, EDTA and MR-A, p. 365–368. *In* Proceedings of the second International Conference on Boar Semen Preservation. Beltsville, Md.
25. **Sakai, K., K. Watanabe, K. Masuda, M. Tsuji, K. Hasumi, and A. Endo.** 1995. Isolation, characterisation and biological activities of novel triprenyl phenols as pancreatic cholesterol esterase inhibitors produced by *Stachybotrys sp.* F-1839. J. Antibiot. (Tokyo) **48:**447–456.
26. **Samson, R. A., E. S. Hoekstra, J. Frisvad, and O. Filtenborg.** 1995. Introduction to food-borne fungi. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
27. **Smibert, R. M., and N. R. Krieg.** 1994. Phenotypic characterization, p. 607–654. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
28. **Sorenson, W. G., D. G. Frazer, B. B. Jarvis, J. Simpson, and V. A. Robinson.** 1987. Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra*. Appl. Environ. Microbiol. **53:**1370–1375.
29. **Sutcliffe, I.** 1994. The lipoteichoic acids and lipoglycans of gram positive bacteria: a taxonomic perspective. Syst. Appl. Microbiol. **17:**467–480.
30. **Taylor, M. J., C. Lafarge-Frayssinet, M. I. Luster, and C. Frayssinet.** 1991. Increased endotoxin sensitivity following T-2 toxin treatment is associated with increased absorption of endotoxin. Toxicol. Appl. Pharmacol. **109:**51–59.
31. **Thai, J. H., and J. J. Pestka.** 1988. Synergistic interaction between the trichothecene T-2 toxin and *Salmonella typhimurium* lipopolysaccharide in c3H/heN and C3H/Hej mice. Toxicol. Lett. **44:**191–200.
32. **Verhoeff, A. O., E. S. Hoekstra, R. A. Samson, B. Brunekreef, and J. H. van Wijnen.** 1994. Fungal propagules in house dust: comparison of analytical methods, p. 49–63. *In* R. A. Samson, B. Flannigan, M. E. Flannigan, A. P. Verhoeff, O. C. G. Adan, and E. S. Hoekstra (ed.), Health implications of fungi in indoor environments. Elsevier, Amsterdam, The Netherlands.
33. **Williams D.** 1994. 1,3-β-D-Glucans, p. 83–86. *In* R. Rylander and R. Jakobs (ed.), Organic dust, exposure, effects and prevention. Lewis Publishers, London, U.K.