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Analysis of *Actinobacteria* from mould-colonized water damaged building material

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A B S T R A C T

Mould-colonized water damaged building materials are frequently co-colonized by actinomycetes. Here, we report the results of the analyses of *Actinobacteria* on different wall materials from water damaged buildings obtained by both cultivation-dependent and cultivation-independent methods. *Actinobacteria* were detected in all but one of the investigated materials by both methods. The detected concentrations of *Actinobacteria* ranged between 1.8×10^4 and 7.6×10^7 CFU g^{-1} of investigated material. A total of 265 isolates from 17 materials could be assigned to 31 different genera of the class *Actinobacteria* on the basis of 16S rRNA gene sequence analyses. On the basis of the cultivation-independent approach, 16S rRNA gene inserts of 800 clones (50%) were assigned to 47 different genera. Representatives of the genera *Streptomyces*, *Amycolatopsis*, *Nocardiopsis*, *Saccharopolyspora*, *Promicromonospora*, and *Pseudonocardia* were found most frequently. The results derived from both methods indicated a high abundance and variety of *Actinobacteria* in water damaged buildings. Four bioaerosol samples were investigated by the cultivation-based approach in order to compare the communities of *Actinobacteria* in building material and associated air samples. A comparison of the detected genera of bioaerosol samples with those directly obtained from material samples resulted in a congruent finding of 9 of the overall 35 detected genera (25%), whereas four genera were only detected in bioaerosol samples.

Introduction

The visible colonisation of water damaged building materials by microorganisms is often associated with the growth of moulds. Hence, microbiological investigations are focused on these eukaryotic microorganisms. Consequently, *Aspergillus versicolor*, *Stachybotrys chartarum* or *Penicillium chrysogenum* have been described as the predominant species and bio-indicators for water damage in building materials [10]. In addition to the material damage caused by microbial growths, they are associated with several negative health effects, such as coughing, wheezing, asthma, airway infections, tiredness and headaches [8,13,50,53]. Until now, however, no clear proof has been provided for a causal connection of the fungal species present, or their metabolites, and the reported negative health effects [54].

Apart from moulds, several authors [2,17,30,38] have reported co-colonisation of water damaged materials by actinomycetes. For example, Lorenz et al. [31] showed the presence of actinomycetes in 61% of 561 materials examined. They detected members of the genera *Nocardia*, *Nocardiopsis*, *Streptomyces*, *Pseudonocardia* and *Promicromonospora*. Recently, Suihko et al. [52] also reported the presence of actinobacteria in 29% of 52 materials investigated. These observations confirmed earlier results [4,6,38]. In addition, Hyvärinen et al. [17] detected members of the genera *Mycobacterium*, *Corynebacterium*, *Rhodococcus*, *Microbacterium* and *Arthrobacter*.

Some *Actinobacteria* are known as biological agents that cause infections or allergic reactions [28,34]. For example, early investigations [22,28,36] showed that *Streptomyces albus*, *Saccharopolyspora rectivirgula* or *Mycobacterium immunogenum* might cause a hypersensitivity pneumonitis. Due to the potential for infection of animals or humans, McNeil and Brown [34] described different *Nocardia* species as relevant for health. In addition, *Streptomyces* and *Mycobacterium* isolates from the indoor environment have been shown to produce cytotoxic metabolites [3]. Toxicological analyses with these strains, for example, on mouse and human macrophages and on a mouse model, also showed cytotoxic effects and an inflammatory response [14,15,20,21,43].

To date, neither the communities of *Actinobacteria* in water damaged building materials nor their impact on exposed inhabitants have been characterised in detail. Here, we report on the results of a comprehensive analysis of *Actinobacteria* from water damaged building materials.

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Table 1
Investigated material samples with the different parameters obtained.

Sample no.	Object	Year of building	Material type	Room type, water damage	Estimated age of water damage	Room temperature	Humidity[a]	pH-value[b]
P1	01	1900	Plaster	Cellar fault, outer wall	Old	9.9 °C	100%	7.0
P2	06	1971	Plaster	Ground floor, kitchen, but damage in bathroom	Old	21.9 °C	88.3%	6.3
P3	07	n.k., old building	Plaster	First floor, kitchen, outer wall, crack of facade	Presumably old	21.1 °C	99.7%	8.0
P4	08	1991	Plaster	Between corridor and bathroom, Leakage next to the shower	Presumably <2 years	19.0 °C	97.9%	8.4
P5	09	1920	Plaster	Ground floor, bedroom, outer wall, presumed hydro-thermal effects	Very old	14.7 °C	72.1%	7.5
P6	11	1960s	Plaster	Nursery, presumed damage of drainage tube	Presumably <2 years	21.2 °C	66.8%	6.8
P7 A/B	12A/B	1934	Plaster	First floor, nursery outer wall, crack of facade	<2 years	24.3–24.8 °C	93.7–96.7%	7.0
P8 A/B	15A/15B	1900	Plaster	Basement, cellar, packing	Very old	15.7 °C	90.2%	7.4
P9	16	ca. 1900	Plaster	Basement, cellar, outer wall, packing	Very old	15–17 °C	>90%	n.d.
S1	03	1980	Styrofoam	Basement, bedroom, burst pipe	<2 years	16.9 °C	95.2%	6.7
S2 A/B	13A/13B	2005	Styrofoam	Ground floor, office, flooring	New building	20.1–21.1 °C	First 93% Later 39%	7.0
M1	02	1982	Insulation wool	Basement, outer wall, hydro-thermal effects	<2 years	13–15 °C	40–60%	9.0
M2 A/B	04/04A	1954	Mineral wool	Nursery first floor Hydro-thermal effects	Presumably old	16.8 °C	59.7%	11.3
M3	05	1971	Mineral material	Ground floor, bathroom, burst pipe	Old	20.4 °C	65.5%	7.1
W1	14	1960	Wallpaper	Basement, cellar, packing	>2 years	22.1 °C	95.9%	7.46
L1	10	Before 1800	Loam rendering	Ground floor, bathroom	Very old	18.8 °C	82.9%	9.3

n.k., not known; n.d., not determined.
[a] pH-values were measured in the laboratory, using a pH-electrode as well as pH-indicator paper.
[b] Humidity was measured on-site, using a measuring instrument (Almemo 2290-2) with a thermistor and sensor for moisture (FH A 646.1, Ahlborn, Holzkirchen).

Materials and methods

Material sampling

A total of 19 mould contaminated building materials were analysed from 16 different objects. Samples were collected in Germany from February 2006 to September 2007, and were distributed throughout the period. Materials were selected on the basis of the most influential parameters for microbial growth. Therefore, all the different parameters for the sampling sites were chosen to overcome limitations regarding the detection of *Actinobacteria*. Additionally, only mould contaminated and moisture damaged materials were taken for investigation because the growth of microorganisms depends to the highest degree on the availability of water, and the lowest water activity values (a_w ~0.65) are necessary to initiate microbial growth [12]. For this reason, no undamaged and dry material was investigated as a "negative control" because it could be expected that no, or only occasionally very few, actinobacteria would be detected. Furthermore, Górny [12] described *Actinobacteria* species as tertiary colonizers of building materials (after different fungi species) because of the necessarily high water content, and a study by Lorenz et al. [31] showed the sole occurrence of bacteria in only 1.8% of the analysed building material. Even regarding health relevance, investigation by Jaakkola et al. [18] showed that the risk of asthma was related to the presence of visible mould and/or mould odour in the workplace, but not to water damage or damp stains alone.

Details on material types, age of the houses, age of and reason for damage, as well as temperatures, dampness and pH-values are given in Table 1. Samples were restricted to houses were health problems had been reported by the inhabitants. Samples were taken from building material with ethanol (70%) cleaned tools. Materials were homogenised, chopped and mixed for 10 min in glass receptacles. In four rooms, bioaerosol samples were collected in parallel. Bioaerosols from 100 to 200 L of room air were collected by impaction using the MAS 100 (Merck, Darmstadt) on three different solid media: actinomycetes isolation agar (Difco, USA), mineral agar according to Gauze et al. [11], and brain heart infusion agar (Difco, USA).

Isolation

Before cultivation, cells were collected in suspension from material by shaking (15 min at 500 rpm) the material in a solution (10-fold by weight) of 0.9% NaCl and 0.01% Tween 80. Subsequently, the cell suspensions were diluted in a tenfold dilution series up to 10^5. From all dilutions, three agar plates were inoculated by plating 0.1 mL of the corresponding suspension on each of the following media: actinomycete isolation agar (Difco, USA), mineral agar according to Gauze et al. [11], brain heart infusion agar (Difco, USA), casein mineral agar [1] and tryptone soy agar (Becton Dickinson, Heidelberg). For inhibition of moulds, 0.1 g L^{-1} natamycin-concentrate (86–93%, AddFood, Ascheberg) was added. All colonies showing different appearances were quantified by counting and then transferred to another agar plate containing subcultivation media. For subcultivation, four different media, ISP-2 agar, ISP-3 agar [49], M79 agar [40] and brain heart infusion agar (Difco, USA), were used. All plates were incubated for 10–21 days at 28 °C.

From all isolates with morphological and microscopic similarities only one "reference isolate" was chosen and studied in more detail. These bacterial isolates were stored at −20 °C and −80 °C in 0.5 mL newborn bovine serum (Bio Wittaker™, Belgium). Colony forming units (CFU) per gram fresh weight were calculated from

the numbers of all different colonies that were determined as actinobacterial isolates.

DNA extraction and PCR amplification

DNA extraction and PCR amplification from bacterial DNA of the isolates

For genomic DNA extraction from objects 1 to 4 (samples P1, M1, S1, M2; Table 1), a loopful of bacterial cells was transferred into a reaction tube (1.5 mL). After disruption of cells by a 1 min bead-beating step (Retsch, Haan) with 1 g of 0.1 Ø Zirconia beads (Carl Roth GmbH + Co, Karlsruhe) at maximum speed, the DNA extraction steps were performed with the GenElute™ Plant Genomic DNA Kit (Sigma), following the instructions of the manufacturer. The 16S rRNA genes were analysed as described by Kämpfer et al. [26]. DNA extraction and PCR-amplification of isolates from objects 5 to 16 (Table 1) were carried out by SMB, Services in Molecular Biology, Rüdersdorf.

DNA extraction and PCR amplification from building material samples

Total DNA was extracted directly from 0.05 to 0.5 g building material using the FastDNA® Spin Kit for soil (MP Biomedicals), following the manufacturer's instructions. For each DNA extraction, a negative control was carried out to ensure the purity of all solutions in the extraction kit. The successfully extracted DNA was used for further PCR and cloning analysis.

PCR was performed in a total volume of 50 μL, prepared in parallel using universal bacteria primers (27f, 1492r) targeting the 16S rRNA gene [29,59]. The PCR assay contained 5.0 μL PCR-buffer, 4 μL $MgCl_2$ (25 mM), 5 μL dNTPs (2 mM), 1 μL of each primer (10 μM, MWG Biotech, Ebersberg, Germany), 31.6 μL RNAse and DNAse free water (Invitrogen, Carlsbad, CA), 0.2 μL BSA (20 mg mL^{-1}), 0.2 μL Taq-polymerase (5 U μL^{-1}) (all MBI Fermentas, St.Leon Rot) and 2 μL DNA extract. PCR was carried out in a Thermocycler (My Cycler™, BioRad, München, Germany) with an initial denaturation step at 95 °C for 3 min, followed by 29 cycles of 1 min at 94 °C, 45 s at 57.3 °C, and 2 min at 72 °C, followed by a final extension at 72 °C for 30 min. PCR was purified by QiaQuick PCR Purification Kit (Qiagen, Hilden) and quantified photometrically (Ultrospec 4000, Amersham Biosciences, Freiburg).

Due to the low amounts of PCR products from the DNA of samples M1 and M2, a nested PCR was performed. First, a PCR using primers 616v [AGAGTTTGATYMTGGCTCAG] and 118r [GGGTTYCCCCATTCGG] was prepared. The reaction mixture of 25 μL contained 2.5 μL PCR-buffer, 3 μL $MgCl_2$ (25 mM), 2.5 μL dNTPs (2 mM), 0.5 μL of each primer (10 μM, MWG Biotech, Ebersberg, Germany), 16.2 μL RNAse and DNAse free water (Invitrogen, Carlsbad, CA), 0.2 μL BSA (20 mg mL^{-1}), 0.1 μL Taq-polymerase (5 U μL^{-1}) (all MBI Fermentas, St.Leon Rot) and 1 μL DNA extract. PCR was carried out in a Thermocycler (My Cycler™, BioRad, München, Germany) with an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 45 s at 57.3 °C, and 2 min at 72 °C, followed by a final extension at 72 °C for 15 min. Amplicons were purified by the QiaQuick PCR Purification Kit (Qiagen, Hilden) and subsequently aliquots were used in a PCR, as described above. A negative control analysis for PCR, containing nuclease free water instead of DNA, was always carried out.

Cloning analysis and screening of generated clones by PCR

For construction of clone libraries, the cloning kit Promega pGEM-T® Vector Systems (Maison, USA) was used according to the manufacturer's instructions. A total of 100 white colonies for each sample were randomly picked and incubated overnight at 37 °C on LB agar containing ampicillin (100 μg mL^{-1}), X-Gal (80 μg mL^{-1}), and IPTG (100 mM) [42]. Inserts were re-amplified to screen all generated clones for affiliation to actinomycetes with two different *Actinobacteria* specific primer systems.

The first primer system, Com2xf (5′-AAACTCAAAGGAATTGACGG-3′) [48, modified] and Ac1186 (5′-CTTCCTCCGAGTTGACCC-3′, developed in our group) was prepared to detect most actinobacteria. The reaction mixture of 25 μL contained 2.5 μL PCR-buffer, 2 μL $MgCl_2$ (25 mM), 2.5 μL dNTPs (2 mM), 0.5 μL of each primer (10 μM, MWG Biotech, Ebersberg, Germany), 16.4 μL RNAse and DNAse free water (Invitrogen, Carlsbad, CA), 0.2 μL BSA (20 mg mL^{-1}) and 0.1 μL Taq-polymerase (5 U μL^{-1}) (all MBI Fermentas, St.Leon Rot). The reaction mixture was added directly to cells of the clones. PCR was also carried out in a Thermocycler (My Cycler™, BioRad, München, Germany) with an initial denaturation step at 95 °C for 3 min, followed by 25 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 15 min. The second PCR using the primer system SC-Act235-aS-20/SC-Act878-aA-19 [51] was carried out to increase the amount of detectable *Corynebacteridae*.

The PCR assay of 25 μL contained 2.5 μL PCR-buffer, 2.5 μL $MgCl_2$ (25 mM), 2.5 μL dNTPs (2 mM), 0.5 μL of each primer (10 μM, MWG Biotech, Ebersberg, Germany), 16.35 μL RNAse and DNAse free water (Invitrogen, Carlsbad, CA), 0.2 μL BSA (20 mg mL^{-1}) and 0.15 μL Taq-polymerase (5 U μL^{-1}) (all MBI Fermentas, St.Leon Rot). The reaction mixture was also added directly to cells of the clones. PCR was performed with an initial denaturation step at 95 °C for 4 min, followed by 25 cycles of 30 s at 95 °C, 1 min at 70 °C, and 2 min at 70 °C, followed by a final extension at 72 °C for 15 min. A control PCR with PCR reagents was always carried out. The PCR products were visually evaluated by 1% agarose gel electrophoresis with ethidium bromide staining. All "positive" detected clones, meaning a PCR product with at least one primer system, were recultured in LB broth, and aliquots were preserved at −80 °C in 99% glycerol in a 1:3 mixture [44].

Sequencing and analysis of the results

Sequencing and analysis of the 16S rRNA gene sequences of the isolates

Sequencing of the 16S rRNA genes of the isolates was carried out by Services in Molecular Biology (Rüdersdorf). The sequences were performed and aligned by comparison with similar sequences using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [55]. Similarity searches against the NCBI database were carried out using a BLAST search (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment with type strains of the detected genera, as well as genetic distance calculations (distance options according to the Kimura 2-parameter model) of the data were also performed using MEGA 4.0. Phylogenetic trees were constructed by the neighbour-joining method using bootstrap values based on 1000 replications. The 16S rRNA gene sequences of isolates and clones reported in the present study were deposited in the GenBank database. Accession numbers are given in the phylogenetic trees (supplementary Figs. S1–S8) and Table 2 under the following accession numbers GU573980–GU574174 and GU594673–GU594680.

Sequencing and phylogenetic analysis of 16S rRNA gene sequences from clone-inserts

The clone colonies were dispatched on LB agar plates to Services in Molecular Biology (Rüdersdorf), where sequencing was carried out. For sequencing, the M13f or M13r sequencing primer (Invitrogen, Carlsbad, CA) was used, resulting in a sequence length of approximately 400 bp. Similarity searches against the NCBI database were performed using a BLAST search (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed only with 16S rRNA gene sequences >1300 bp by the

Table 2
Isolate and clone sequence most closely related to bacteria assigned to risk group 2, according to TRBA 466.

Isolate/clone (accession no.)	Most closely related species	Sequence similarity[a]	Accession no.	Risk group
02-Gi-019 (GU574174)	*Corynebacterium amycolatum*	99.40%	X82057	2
01-Gi-005 (GU574063)	*Mycobacterium flavescens*	98.40%	X52932	2
05-Be-043 (GU574173)	*Mycobacterium wolinskyi*	98.80%	Y12873	2
06-St-023 (GU574127)	*Nocardiopsis dassonvillei* subsp. *albirubida*	100%	X97882	2
07-St-010 (GU574130)	*Nocardiopsis dassonvillei* subsp. *albirubida*	100%	X97882	2
05-St-001 (GU574122)	*Nocardiopsis dassonvillei*	100%	X97886	2
05-Be-009 (GU574123)	*Nocardiopsis dassonvillei*	99.90%	X97886	2
P2-S-DW11-16 clone (GU574058)	*Propionibacterium granulosum*	99.50%	AJ003057	2

[a] Multiple alignment, distances (distance options according to the Kimura 2-parameter model) were performed by using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [55].

neighbour-joining method, using bootstrap values based on 1000 replications (together with the sequences of the isolates).

Results

Concentration

To investigate the actinobacterial communities in water damaged buildings, 19 material samples and 4 bioaerosol samples from 16 objects were investigated. *Actinobacteria* were detected in 18 out of 19 investigated material samples by cultivation-based and/or by molecular methods (Table 1). Bacterial growth could not be detected in Styrofoam sample S2B. The concentration of cultivable *Actinobacteria* in bioaerosol samples varied between 4.7×10^2 CFU m^{-3} and 8.5×10^2 CFU m^{-3}. The concentration of *Actinobacteria* in investigated materials varied between 1.8×10^4 CFU g fw^{-1} (gram fresh weight) and 3.4×10^7 CFU g fw^{-1} in Styrofoam samples, between 7.8×10^5 CFU g fw^{-1} and 7.6×10^7 CFU g fw^{-1} in plaster samples, and between 4.4×10^4 CFU g fw^{-1} and 4.1×10^6 CFU g fw^{-1} in mineral material. The concentration detected in wallpaper and loam rendering was 1.7×10^5 CFU g fw^{-1} and 3.7×10^5 CFU g fw^{-1}, respectively (Fig. 1).

Isolates

From all samples (material and bioaerosol samples), a total of 419 isolates were obtained. Multiple isolated bacteria were grouped according to the colony and cell morphology, as well as Gram staining. From one representative isolate of each group ($n = 334$), the 16S rRNA gene was sequenced. Based on 16S rRNA gene sequence analyses, 293 isolates were assigned to the class *Actinobacteria* and 17 isolates to the *Alphaproteobacteria* or *Firmicutes*. Some isolates ($n = 24$) could not be identified because subcultivation failed. The actinobacterial isolates were representatives of 35 different genera, of which species of *Streptomyces* ($n = 94/32.1\%$), *Amycolatopsis* ($n = 20/6.8\%$), *Nocardiopsis* ($n = 20/6.8\%$), *Nocardia* ($n = 15/5.1\%$), *Pseudonocardia* ($n = 21/7.2\%$), *Saccharopolyspora* ($n = 13/4.4\%$) and *Promicromonospora* ($n = 16/5.5\%$) were found most frequently. Seven isolates were assigned to the genus *Mycobacterium*. In total, seven species closely related to bacteria of risk group 2 were detected (Table 2).

Clone libraries

In addition to the cultivation-based approaches, the *Actinobacteria* populations were also analysed in this study by the generation of 16S rRNA gene clone libraries. From 1587 generated clones, approximately 50% ($n = 800$) showed specific PCR products using *Actinobacteria* specific primers. Depending on the investigated material, the proportion of "*Actinobacteria* positive clones" varied between 8.7% and 88.5% (Fig. 2). After partial sequence analysis, 47 different genera of the class *Actinobacteria* were detected. The most abundant genera were *Amycolatopsis* ($n = 134/16.8\%$), *Arthrobacter* ($n = 48/6.0\%$), *Jiangella* ($n = 33/4.1\%$), *Nesterenkonia* ($n = 44/5.5\%$), *Promicromonospora* ($n = 59/7.4\%$), *Pseudonocardia* ($n = 121/15.1\%$),

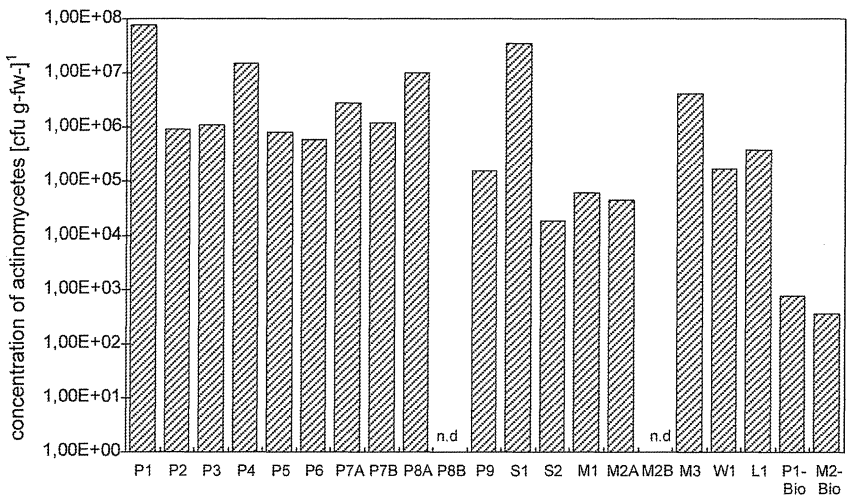

Fig. 1. Concentrations of *Actinobacteria* in CFU g^{-1} fw (m^{-3}) of investigated material samples (17) and bioaerosol samples (2). Values represent results of single analyses. n.d., not determined.

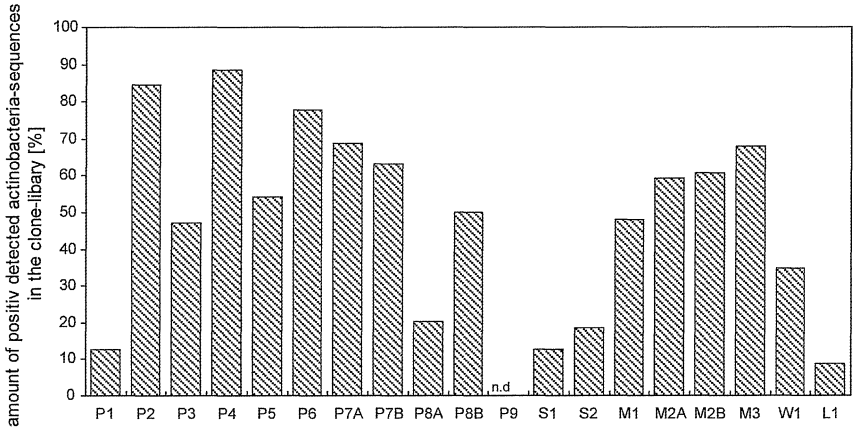

Fig. 2. Amount of detected actinobacteria clones (%) from analysed material samples. Values represent results of single analyses. n.d., not determined.

	genera	P1		P2		P3		P4		P5		P6		P7A		P7B		P8A		P8B*		P9†		S1		S2		M1		M2A		M2B*		M3		W1		L1		Σ detected in 19 samples	Σ only isolation	Σ only cloning	double match
		i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c	i	c				
1	*Actinoalloteichus*																																							1	0	1	0
2	*Actinomycetospora*																																							2	0	2	0
3	*Actinopolymorpha*																																							5	0	5	0
4	*Aeromicrobium*																																							3	0	3	0
5	*Agrococcus*																																							3	1	2	0
6	*Agromyces*																																							1	0	1	0
7	***Amycolatopsis***																																							**12**	**2**	**2**	**8**
8	***Arthrobacter***																																							**12**	**1**	**9**	**2**
9	*Blastococcus*																																							3	0	3	0
10	*Brachybacterium*																																							4	1	3	1
11	*Brevibacterium*																																							8	3	4	1
12	*Citricoccus*																																							2	2	0	0
13	*Clavibacter*																																							1	0	1	0
14	*Conexibacter*																																							2	0	2	0
15	*Corynebacterium*																																							1	1	0	0
16	*Georgenia*																																							1	1	0	0
17	*Glaciibacter*																																							2	0	2	0
18	*Goodfellowiella*																																							1	0	1	0
19	*Isoptericola*																																							2	2	0	0
20	***Jiangella***																																							**12**	**2**	**7**	3
21	*Klugeriella*																																							1	0	1	0
22	*Kocuria*																																							3	2	1	0
23	*Kribbella*																																							2	1	1	0
24	*Leifsonia*																																							1	0	1	0
25	*Lentzea*																																							5	2	1	2
26	*Leucobacter*																																							1	1	0	0
27	*Microbacterium*																																							8	3	4	1
28	*Microcella*																																							1	0	1	0
29	*Micrococcus*																																							3	3	0	0
30	*Microlunatus*																																							2	1	1	0
31	*Micromonospora*																																							4	3	1	0
32	*Murinocardiopsis*																																							2	2	0	0
33	*Mycetocola*																																							1	0	1	0
34	*Mycobacterium*																																							6	4	1	1
35	*Nesterenkonia*																																							3	0	3	0
36	***Nocardia***																																							**11**	**9**	**0**	**2**
37	*Nocardioides*																																							6	1	1	4
38	***Nocardiopsis***																																							**11**	**4**	**4**	**3**
39	*Oerskovia*																																							1	1	0	0
40	*Ornithinicoccus*																																							1	1	0	0
41	*Prauserella*																																							2	2	0	0
42	***Promicromonospora***																																							**13**	**4**	**3**	**6**
43	*Propionibacterium*																																							1	0	1	0
44	*Propionicicella*																																							1	1	0	0
45	***Pseudonocardia***																																							**17**	**0**	**6**	**11**
46	*Rathayibacter*																																							2	0	2	0
47	*Rhodococcus*																																							3	2	0	1
48	*Ruania*																																							1	0	1	0
49	*Rubrobacter*																																							1	0	1	0
50	*Saccharomonospora*																																							2	0	2	0
51	***Saccharopolyspora***																																							**12**	**2**	**7**	**3**
52	*Solirubrobacter*																																							1	0	1	0
53	*Stackebrandtia*																																							3	0	3	0
54	***Streptomyces***																																							**17**	**7**	**4**	**6**
55	*Tsukamurella*																																							1	1	0	0
56	*Umezawaea*																																							1	0	1	0
57	*Yania*																																							1	0	1	0
58	*Yonghaparkia*																																							3	0	3	0
	Σ detected genera	17		17		12		11		6		14		14		12		16		16		2		14		9		18		12		13		14		9		6		232 (100%)	73 (31,5%)	106 (45,7%)	55 (23,7%)

Fig. 3. Abundance of clones and isolates. Distribution of the clones and isolates in taxonomic genera and their abundance in the individual samples are displayed in columns 1–19. The light grey shading indicates a match via isolation; the dark grey shading indicates a match by cloning analyses in the sample belonging to the respective genera. White cells indicate no match. Column 20 shows the number of samples in which the different genera were detected. Columns 21 and 22 show the number of samples in which different genera were detected only by isolation or cloning analyses, whereas in column 23 the number of samples is listed in which genera were detected by both cultivation and cloning analyses; *Only investigated by cloning analyses; †only investigated by isolation analyses; i = isolation; c = cloning analyses.

Table 3
Comparison of detected genera in material and bioaerosol samples, number of isolates within the genera with sequence similarities > 99% are given in parentheses[a].

Bioaerosol sample	Material sample	Material and bioaerosol sample
Indoor object 1 (sample P1)		
Janibacter	*Amycolatopsis*	*Arthrobacter*
Kocuria	*Brevibacterium*	*Nocardiopsis* (1)
Pseudonocardia	*Jiangella*	*Rhodococcus*
	Kribella	*Streptomyces*(2)
	Microbacterium	
	Micrococcus	
	Mycobacterium	
	Nocardia	
	Nocardia	
	Ornithinimicrobium	
	Promicromonospora	
	Tsukamurella	
Indoor object 2 (sample M1)		
Arsenicicoccus	*Brevibacterium*	*Nocardiopsis*
Kytococcus	*Citricoccus*	*Saccharopolyspora*
	Corynebacterium	*Streptomyces*(1)
	Kocuria	
	Micrococcus	
	Micromonospora	
	Pseudonocardia	
Indoor object 3 (sample S1)		
Actinomadura	*Amycolatopsis*	*Brevibacterium*(1)
Micrococcus	*Isoptericola*	*Nocardia*(1)
Rhodococcus	*Jiangella*	*Streptomyces*(1)
	Microbacterium	
	Mycobacterium	
	Nocardioides	
	Promicromonospora	
	Pseudonocardia	
Indoor object 4 (sample M2)		
	Pseudonocardia	*Micrococcus*(1)
		Streptomyces

[a] Column 1 shows the indoor environment from where the material and bioaerosol samples were taken and listed the genera only detected in bioaerosol samples; column 2 shows the genera only detected in material samples; column 3 shows the genera which were detected in both the bioaerosol sample and the material sample.

Saccharopolyspora ($n = 78/9.8\%$) and *Streptomyces* ($n = 83/10.4\%$).

Comparison of cultivation-dependent and cultivation-independent methods

Overall, 58 different genera were detected within the 19 investigated water damaged samples (Fig. 3). Depending on the investigated material, the number of detected genera varied between 2 and 17 (mean = 12) per material. The number of the "genera matches" in all samples was 232. From these, 31.5% ($n = 73$) and 45.7% ($n = 106$) were detected exclusively by cultivation or by cloning analysis, respectively. In total, 23.7% ($n = 55$) of the matches were detected by both methods in parallel. Independent of the method, the most frequently found genera were *Pseudonocardia* (detected in 17 samples out of 19 samples, 17/19), *Streptomyces* (17/19), *Amycolatopsis* (12/19), *Saccharopolyspora* (12/19), and *Promicromonospora* (13/19).

Comparison of isolates from material and bioaerosol samples

To investigate the actinobacterial communities in indoor air, four bioaerosol samples were investigated by the cultivation-based approach. All detected genera are listed in Table 3. Altogether, nine genera were detected both in bioaerosol and material samples, and four genera, *Actinomadura*, *Arsenicicoccus*, *Janibacter* and *Kytococcus* were only detected in bioaerosol samples. Comparison of the detected genera in material and corresponding bioaerosol samples resulted in a room dependent congruent finding of 20–66% at the genus level. 16S rRNA gene sequence similarities > 99% for isolates found both in bioaerosol samples and the corresponding material samples were detected in 8 out of 28 isolates (Table 3).

Discussion

Actinobacteria were detected in all but one of the investigated materials by both cultivation-dependent and cultivation-independent methods. In previous studies, *Actinobacteria* were reported in 18–70% of the investigated materials, dusts or bioaerosols of water damaged buildings, respectively [17,31,37,52]. All these results indicate the ubiquitous distribution of *Actinobacteria* in water damaged indoor environments.

Concentrations of *Actinobacteria*, cultivable using the applied media, varied between 1.8×10^4 and 3.4×10^7 CFU g^{-1} fresh weight in, for example, Styrofoam samples and to $\sim 8 \times 10^7$ CFU g^{-1} fresh weight in investigated plaster samples (Fig. 1). In comparison, investigations from Suihko et al. [52] showed approximate counts of filamentous actinobacteria and/or thermoactinomyctes of 10^2–10^3 CFU g^{-1} fresh weight in gypsum board, wallpaper or paint and plaster crumbs, and only higher counts of 10^4–10^6 CFU in insulator wool samples. Higher values of actinobacterial concentrations from our studies are similar to those found by FISH analyses in natural bulk soil habitats [60]. Therefore, the investigated building materials seem to offer similar good growth conditions for *Actinobacteria* species as soil. In contrast to soils, where *Actinobacteria* are assumed to represent only about 4% of the total bacterial community [19], investigations of clone libraries revealed their predominance in the studied materials. The amounts of detected actinobacterial 16S rRNA genes varied between 12.6 and 88.5% in clone libraries (Fig. 2). This has also been shown by Rintala et al. [42] in investigations of dust samples from an indoor environment with water damage, and they showed that 24% of the 16S rRNA gene sequences obtained in generated clone libraries could be assigned to *Actinobacteria*.

Quite a high variety of the detected *Actinobacteria* was observed, with 58 different bacterial genera (Fig. 3) from 11 different (sub)-orders (*Corynebacterineae*, *Frankineae*, *Glycomycineae*, *Micrococcineae*, *Micromonosporineae*, *Propionibacterineae*, *Pseudonocardineae*, *Rubrobacterineae*, *Solirubrobacterales*, *Streptomycineae* and *Streptosporangineae*). In relation to case reports [2,4,30,38,42,52,58], almost all of the genera detected in these studies were also found in our comprehensive approach. A comparison with the results of the study from Suhiko et al. [52], who investigated only filamentous *Actinobacteria* and thermoactinomycetes from 52 water damaged building materials using a cultivation-dependent approach, showed that much more genera were detectable in the present study. While these authors detected five different actinobacterial genera by cultivation of 122 isolates, our results from cultivation gave 31 different genera from 265 isolates from building material samples. These findings from Finland probably resulted from lower detectable concentrations of investigated materials, as well as lower occurrences (29%) of *Actinobacteria* themselves. Furthermore, in the present study, 23 different genera were additionally detected by the cultivation-independent approach.

Some genera often detected in our study, for example, *Amycolatopsis* and *Jiangella*, have not been described as colonizers of water damaged indoor material to date. In addition, it was an interesting finding that although most of the isolates could be assigned to already described species, several of them could not be clearly assigned to any known species or even genera, and hence novel species and genera have been proposed, such as

for example, *Pseudonocardia parietis* [45], *Citricoccus parietis* [46], *Prauserella muralis* [47], *Promicromonospora umidemergens* [33], *Brevibacterium sandarkinum* [23], *Microlunatus parietis* [25], *Kytococcus aerolatus* [24], and *Murinocardiopsis flavida* [27].

Genera detected in nearly 60% (≥11) of the investigated ($n = 19$) materials were *Arthrobacter* (12/19; detected in 12 samples out of 19 investigated samples), *Promicromonospora* (13/19), *Pseudonocardia* (17/19), *Jiangella* (12/19), *Amycolatopsis* (13/19), *Nocardia* (11/19), *Saccharopolyspora* (11/19), *Nocardiopsis* (12/19), and *Streptomyces* (17/19) (supplementary material, Figs. S1–S8). These genera may serve as actinobacterial indicators of water damage in buildings, like fungi such as *A. versicolor*, *S. chartarum* or *P. chrysogenum*.

In regard to identifying the most abundant species, 16S rRNA gene sequence similarities to the sequences available in the databases indicate that these species were most closely related to *Amycolatopsis palatopharyngis* (>98.8%) and *Nocardia carnea* (>99.6%), which were both found in nine different materials. *P. parietis* sp. nov. [45] was detected in seven investigated materials. In addition, the species most closely related to *Saccharopolyspora flava* (>97.8%) were detected in six different samples, and *Nocardiopsis* sp. (most closely related to *Nocardiopsis dassonvillei*, *Nocardiopsis dassonvillei* subsp. *albirubida* and *Nocardiopsis synnemataformans*) were also found in six different materials.

In the present study, members of the genus *Streptomyces* were detected in highest concentrations and in nearly all of the investigated materials (17/19), by both the cultivation-dependant and -independent approaches. This is essentially in agreement with earlier studies reporting *Streptomyces* in indoor environments [3,41,43]. Based on 16S rRNA gene analyses, the intrageneric variety of *Streptomyces* isolates was high (the sequence allocations were scattered across the *Streptomyces* tree). Rintala et al. [41] detected 16S rRNA sequences most closely related to *Streptomyces griseus*, *Streptomyces setonii*, *Streptomyces coelicolor*, *Streptomyces virginiae*, *Streptomyces sampsonii* and *Streptomyces rimosus*, and Roponen et al. [43] described *Streptomyces anulatus* isolated from gypsum board. All these results showed a high abundance and high variety of *Streptomyces* species in water damaged materials.

Similar to the high abundance of *Streptomyces*, members of the genus *Pseudonocardia* were detected in 17 out of 19 investigated materials. Lorenz et al. [30,32] described *Pseudonocardia* spp. from a plaster sample attached to some wall paper. In addition to *Streptomyces*, this genus may serve as a further indicator of water damage in an indoor environment. In the present study, the 16S rRNA gene sequences detected from isolates could be assigned to four sequence clusters most closely related to (i) *P. parietis*, (ii) a group of species comprising *Pseudonocardia alni*, *Pseudonocardia carboxydivorans*, *Pseudonocardia antarctica*, and (iii) two sequence clusters most closely related to *Pseudonocardia ammonioxydans*. 16S rRNA gene sequences of investigated clone libraries formed two further clusters which were most closely related to (i) *Pseudonocardia spinosispora* and (ii) *Pseudonocardia petroleophila*.

Similar negative health effects, as described for some *Streptomyces* spp., may be assumed for some *Nocardiopsis* species, which were detected in 12 out of 19 investigated materials. According to the "German" national classification list TRBA 466 [56], *N. dassonvillei* is classified into risk group (RG) 2 (RG 2: biological agents that can cause human disease and might be a hazard to workers). Four isolates were found to be most closely related to the two subspecies of *N. dassonvillei*. Peltola et al. [39] isolated *Nocardiopsis exhalans* (RG 1) from dust and reported cytotoxic effects for sperm cells, and Mehrer et al. [35] showed cytotoxicity of *Nocardiopsis* sp. towards swine kidney cells. Using cloning analyses, the presence of *N. exhalans* was also confirmed in our study but the majority of 20 isolated *Nocardiopsis* spp. were found to be most closely related to *Nocardiopsis synnemataformans* and *N. dassonvillei*. In addition, representatives of genera less frequently detected, for example, *Brevibacterium*, *Corynebacterium*, *Mycobacterium*, *Tsukamurella*, *Actinomadura*, *Microbacterium*, *Rhodococcus* and *Propionibacterium* were detected as well. The detection of species from these genera confirmed earlier investigations [5,38,42,57,58].

In particular, the genus *Mycobacterium* is of interest because several indications exist concerning its relevance in water damaged indoor environments. In this connection, Andersson et al. [5] detected *Mycobacteria* and related genera as major colonizers of a wall in a children's day care centre with visible moisture damage. Torvinen et al. [57] also described different potential pathogenic *Mycobacteria* isolated from water damaged building materials. *Mycobacteria* species are known to be responsible for different negative health effects, such as infections, inflammatory response, production of cytokines, NO and IL6 [16,21] and hypersensitivity pneumonitis [7,9]. In the present study, seven different *Mycobacterium* species were detected. Based on 16S rRNA gene comparison, two isolates were most closely related to *Mycobacterium flavescens* and *Mycobacterium wolinskyi*. Both species are classified as biological agents of risk group 2. Remaining isolates were most closely related to *Mycobacterium moriokaense* ($n = 2$), *Mycobacterium tusciae*, *Mycobacterium septicum* and *Mycobacterium alvei*, which are classified as biological agents of risk group 1.

Infections or allergic reactions can only occur after direct contact with relevant pathogens. To investigate a potential release of bacteria from water damaged material we compared the actinobacterial communities in bioaerosols and the corresponding water damaged materials from four rooms. From 20% to 66% of the detected genera were congruent. In both bioaerosol and material samples, representatives of the genera *Arthrobacter*, *Brevibacterium*, *Kocuria*, *Micrococcus*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus* and *Streptomyces* were detected (Table 3). On the basis of a 16S rRNA gene sequence similarity > 99% from 28 isolates, 8 isolates (28%) from bioaerosols were also found in the material samples. Most of the members of the genera listed above are able to produce aerial mycelia as well as spores which can be released into the air. In conclusion, the present study showed an unexpectedly high number of diverse members of the class *Actinobacteria* in water damaged building material samples. However, possible health effects are not clear at present and deserve further detailed investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.04.006.

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