ELSEVIER

International Biodeterioration & Biodegradation 53 (2004) 13–24

INTERNATIONAL BIODETERIORATION & BIODEGRADATION

www.elsevier.com/locate/ibiod

Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St. Martins church (Greene–Kreiensen, Germany)

Anna A. Gorbushina[a,*], Jeroen Heyrman[b], Thomas Dornieden[a], M. Gonzalez-Delvalle[c], Wolfgang E. Krumbein[a], Leonila Laiz[c], Karin Petersen[a], Cesareo Saiz-Jimenez[c], Jean Swings[b,d]

[a] *AG Geomikrobiologie, ICBM, Oldenburg University, Postfach 2503, Oldenburg 26111, Germany*
[b] *Laboratory for Microbiology, Ghent University, K.L. Ledeganckstraat 35, Gent 9000, Belgium*
[c] *CSIC, Instituto de Recursos Naturales y Agrobiologia (IRNAS), CSIC, Apartado 1052, Sevilla 41080, Spain*
[d] *BCCM/LMG Culture Collection, K.L. Ledeganckstraat 35, Gent 9000, Belgium*

Received 11 November 2002; accepted 16 July 2003

Abstract

Microbial biofilms were massively developing on the surfaces and within the painting layers of mural paintings of a parish church in Lower Saxony, which were exposed and restored in the end of 1970s. The causes of the heavy infections remained unclear. Within the frame of an European research project (ENV4-CT98-0705) these microbial infections were documented and analyzed. By scanning electron microscopy (SEM) and dissecting microscope analysis of mural painting fragments it was shown that the main biofilm formers were microscopic fungi with strong pigment development. Thirty-two fungal and 139 heterotrophic bacterial isolates were obtained by cultivation methods. Most of the fungi (32 isolates) were characterized by morphological methods and nutritional physiology (BIOLOG system) and identified as *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium* and other imperfect fungal genera among which several melanized *Mycelia sterilia*. Representative bacterial strains were analyzed by 16S rDNA sequencing, the majority of bacteria belonged to the genera *Arthrobacter*, *Bacillus* and *Bacillus*-related genera. Isolated strains (both fungal and bacterial) belong to spore formers and thus could have been potentially stimulated to grow only by the transfer to the growth medium. The results of SEM analysis, cultivation experiments and visualization of microbial activity, confirm the hypothesis that the current microbial community is inactive and represent a stagnant microbial community developed after drastic environmental changes caused by an unfortunate conservation treatment.

Keywords: Mural paintings; Subaerial biofilms; Micromycetes; Heterotrophic bacteria; Identification; Biodeterioration analysis

1. Introduction

Subaerial biofilms—microbial growth on atmosphere exposed surfaces—present a unique environment, in which only microorganisms adapted to conditions of limited water availability are able to grow. Bacteria (including actinomycetes), fungi, and lichens represent an important part of the subaerial microbial community. Their growth forms reflect the adaptations acquired to grow in the limiting environment they inhabit. The main growth restrictions are due to constant and unpredictable changes of the environmental conditions on a subaerial surface. Temperature, humidity (or water content), and irradiation are changing in a diurnal or annual fashion and thus pose adjustment problems to all microbes. A special case of subaerial environment is represented by indoor surfaces in unheated or only periodically heated buildings (e.g., churches). In these semi-closed environments the microbial community is enclosed in a mesocosm, partially isolated from the external environment and relatively stable in comparison to out-door locations. In this investigation, the microbial biodiversity on mural paintings of a church in Greene (Lower Saxony, Germany) was studied. Small samples of paint layer were analyzed for the presence of microorganisms by microscopic (dissecting LM and SEM) and cultivation methods. It is generally believed, that with the use of current types of culture media, we are able

* Corresponding author. Tel.: +49-441-798-3393; fax: +49-441-798-3384.
E-mail address: a.gorbushina@uni-oldenburg.de (A.A. Gorbushina).

0964-8305/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibiod.2003.07.003

to cultivate only a small part of the whole microbial population. The more complex the environment, the more difficult it is to cover the whole spectrum of microorganisms present. In this investigation, a broad spectrum of isolation methods and media was employed in order to document and isolate a wide variety of heterotrophic microorganisms thriving on mural paintings. Air of the church was analyzed for the presence and quantity and quality of microorganism propagules. Environmental conditions (wall humidity and temperature) were characterized to complete the picture of an exceptional deterioration case and to compare the sampling points inside the church.

2. Materials and methods

2.1. Site description and sampling

Samples for the study were taken from the mural paintings of St. Martin church in Greene–Kreiensen (Lower Saxony, Germany). The church was built in 1439. The murals were painted after the change from a catholic to protestant use in the year 1582. Later, they were covered by white wash from 1716 to 1977. After the mural paintings had been freshly exposed and restored in 1977, a strong development of biofilms was observed. The growth of biofilm was connected with the development of macroscopically visible greyish-brown colonies of filamentous microorganisms and resulted in a heavy discoloration. Most conspicuous was the development of the biofilm on the western wall (Figs. 1–3), which was probably connected with concrete injections used as a conservation treatment in 1977. Similar biogenic structures and discolorations are also observed on the northern and southern walls, but are less expressed there.

Three samples for cultivation and corresponding subsamples of intact painting layer for SEM analysis were taken from the surface layer of the painting containing discolorations and presumed biological growth. Sampling was undertaken on the 22nd of March 1999, with the help of a sterile glass-fibre brush (for cultivation) or scalpel (SEM) for the samples G4, G9, and G11. These powdery samples were divided between working groups and used for isolation of bacteria and micromycetes as well as for molecular analysis (Gurtner et al., 2000). Sample G4 was taken from dry biofilms of filamentous colonies growing at the west wall of the church (fresco "Adoration of the Magi"), at a height of 1.5 m (height was measured from the floor of the organ-loft). Sample G9 and sample G11 were both taken at the north wall (fresco "Jacobus Major") of conspicuous brown growth spots, at 2.0 and 1.7 m height, respectively. The main difference between the two samples from the northern wall was the structural composition of the paint support, which in sample G11 contained some straw fragments. At sample points G4 and G11, additional samples were taken for SEM analysis. Filamentous structures

Fig. 1. The elevated fungal colonies on the lower part of the western wall as presented in side illumination. Magnification 2×.

Fig. 2. The development of the discoloration is frequently coinciding with a stronger relief of the wall and could thus be misinterpreted as dust accumulations. However, as it was demonstrated by SEM, these accumulations are practically all caused by extended subaerial biofilms dominated by micromycetes.

from the western and the southern wall (samples G1 and G2, respectively) were taken with a sterile syringe needle and immediately aseptically transferred to media. These direct transfer samples were taken from dry biofilms consisting of filamentous colonies and both at 1.5 m level. Sampling was carried out with the help of Dipl.-Rest. B. Retherath (Fa. Ochsenfarth) and under severe restrictions by the conservation authorities, thus sample size and numbers were reduced to the minimum. A second sampling was carried out in May 2000, when small fragments of biofilms (close to sampling point G4 with the most expressed biologenic phenomena) were repeatedly transferred to the media for cultivation.

2.2. Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were air-dried, coated with gold (Balzers Union SCD 030) and

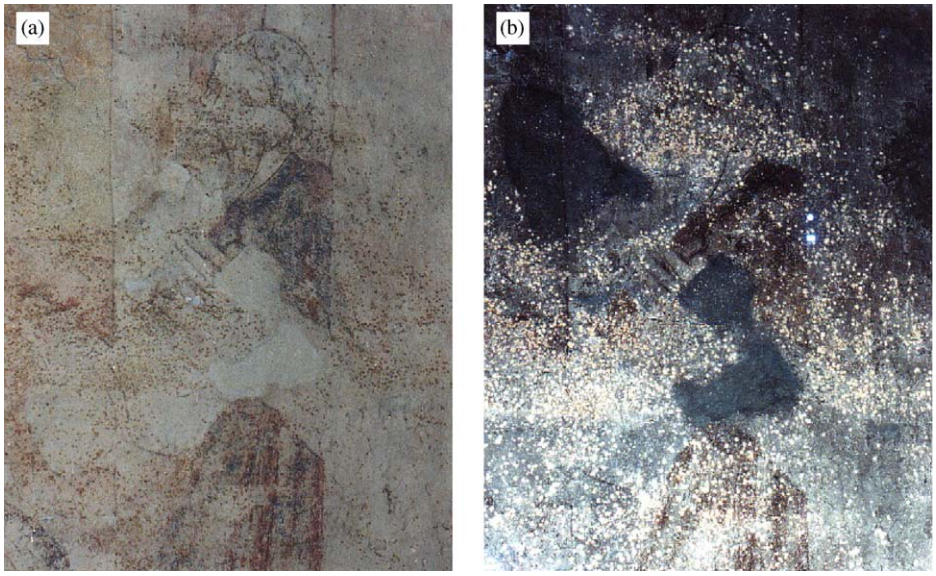

Fig. 3. (a) Fungal mycelium extends over large surface areas with mainly the sporulating parts visible; (b) illumination by UV-light visualizes mycelial structures due to their autofluorescence. Especially important is the obvious absence of growth on new additions to the painting.

examined in Hitachi Scanning Electron Microscope S-3200N. The accelerating voltage was 18–20 kV.

2.3. Cultivation, isolation and characterization of heterotrophic bacteria

For culturing heterotrophic bacteria, the samples were suspended in physiological solution and blended for 1 min using a Stomacher Lab-blender (L.E.D. Techno, Eksel, Belgium). A dilution series was made and plating and incubation were conducted as previously described (Heyrman et al., 1999). Colonies were isolated from plates inoculated with the highest dilutions showing growth. A total of 139 bacterial strains were isolated.

For further characterization and identification of heterotrophic bacteria, the bacterial strains were first studied using fatty acid methyl ester analysis (FAME) (Heyrman et al., 1999). The FAME-clustering, obtained by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) of the Canberra metric coefficients calculated between the fatty acid profiles of strains isolated from 11 samples derived from three European wall paintings (Greene, Herberstein and Carmona), was used as a starting point for further identification of the bacterial isolates. A representative of the major clusters and some ungrouped strains were analyzed by 16S rDNA sequence analysis. Total genomic DNA of the isolates was extracted according to a slightly modified method of Pitcher et al. (1989). 16S rDNA was amplified with eubacterial primers (p27f and 1522r, Edwards et al., 1989) and sequenced as previously described by Heyrman and Swings (2001).

2.4. Cultivation, isolation and characterization of micromycetes

Micromycetes were isolated from sample material, which was aseptically crushed with a mortar, suspended in physiological solution and plated with the help of a Spiral Plater system (spiral systems). Several (nine) different agar media were used for the specific isolation of micromycetes. The following media were prepared: 2% malt agar with 0.03% streptomycin, Czapek–Dox agar with 0.03% streptomycin, PYGV (0.025% pepton, 0.025% yeast extract, 0.025% glucose, Hunter mineral salt solution 20 ml, vitamin solution 10 ml (Staley, 1968)), ECA (meso-erythritol 1%, chloramphenicol 0.05%, Yeast–Nitrogen base 0.67%), skim-milk agar, dichloran rose bengal medium (DRBC, King et al., 1979), PPYG (Gee et al., 1989) and BRII (Bunt and Rovira, 1955) with pH 9.5 and 10.5. Incubation was conducted at 18°C in dim light. A total of 32 fungal strains were isolated representing 11 genera. Special attention was given to the colonies, which were gained by the direct transfer of biofilm fragments from the wall surface to nutrient media (Czapek–Dox agar with 0.03% streptomycin and DRBC). It was supposed that direct isolation represents the autochthonous population causing the observed biodeterioration phenomena and dwelling on the wall for a longer period.

For further characterization and identification of micromycetes, the isolates were studied under the light microscope and identified to generic level according to their morphological characteristics (Domsch et al., 1980). Abilities of the strains to survive conditions of growth on the original mural paintings were tested on media with high pH levels (PPYG, BRII, skim-milk agar). A screen with

RAPD (random amplified polymorphic DNA) was used to distinguish between the isolates morphologically assigned to phialosporic genera (*Acremonium* and microsporic *Fusarium*). Thus the majority of the isolates were characterized to the generic level morphologically, molecularly, and by nutritional physiology. A BIOLOG system for characterization and identification of strains by their ability to utilize different carbon sources was employed.

2.5. Environmental parameters and air analysis

Dampness in the walls was measured in May 2000 with the help of a contact microsensor AMR FH A696 MF with a connected data logger AMR Almemo 2290-8.

Air analysis for propagation units of fungi was carried out with microbiological air sampler MAS-100 (Merck). Fifty liters of air were filtered onto the surface of nutrient media. Czapek–Dox agar with 0.03% streptomycin and DG18 (glycerol containing medium with low water activity) plates were used. At each point the analysis was undertaken in triplicate. Colony counts were estimated after 7 days of incubation.

2.6. Fluorescent dyes and other chemicals for measurement of microbial activity

To determine cell viability a combination of fluorescein diacetate (FDA, F-1303) and propidium iodide (PI, P-1304) was used. The dyes were obtained from Molecular Probes Inc. (Eugene, OR, USA) and visualized by fluorescent microscopy (Axioplan, Zeiss).

3. Results and discussion

3.1. Visual observations, SEM analysis and measurement of microbial activity

All the samples under investigation were characterized by a predominant development of fungal biofilms. The typical discoloration spots were spread over the wall painting surface on all the walls. In some parts (especially in the lower part of the wall above the organ-loft) the biofilms are significantly thicker and developed in a carpet-like fashion. The powdery growth spots consisted of branched predominantly fungal filaments and were recognizably elevated over the wall surface (Fig. 1). The development of the discoloration was frequently coinciding with a stronger relief of the wall, which could be misinterpreted as dust accumulations (Fig. 2). Fungal mycelium extended over large surface areas (Fig. 3a). When the walls were illuminated by UV-light, spreading of the colonies was also detectable in non-discolorated parts (Fig. 3b) due to autofluorescence of the fungal cell wall components (Gramann, 1998). The original subaerial biofilm observed in SEM demonstrated diverse microbial structures that were developing in close contact with the material of the paint layer. Microscopic fungi were predominant as demonstrated by the presence of hyphae abundantly spreading in all samples investigated (Figs. 4–9).

On the western wall (samples G3 and G5, “Adoration of the Magi”, SEM samples corresponding to G4) the biofilm was developed extensively and showed a brownish pigmentation. Filaments of fungi as well as fungal spores were numerous on the surface of the painting layer (Figs. 4a and 5). Thinner filaments, assigned to the growth of actinomycetes, were also frequently observed (Figs. 8 and 9). A significant portion of the microbial structures was spore bearing and more or less the same in all samples (Fig. 5). Bacterial cells were only rarely observed and represented only a small portion of in situ colonization. Fungal hyphae, which grow in close contact with the wall, were the dominant features. Especially interesting was the observation of an extracellular matrix that enhances contact of the biofilm with the substrate and possibly supports the community in its survival under these difficult hostile conditions (Florian, 1997). In general, discolorations and alterations of the paint layer located at the upper part (1.5 m above the floor of the loft) of the western wall, were caused by a complex subaerial microbial community consisting mainly of micromycetes and actinomycetes. The lower part of the western wall (“Adoration of the Magi”, sample G5, 25 cm above the floor) was covered by a thicker biofilm. However, macroscopically no differences could be observed in consistency or color of the brownish powdery dry biofilm. SEM observations have demonstrated the prevailing presence of fungal filaments and sporulation structures (conidiophores) on the surface of the painting layer (Figs. 5 and 6) and in the deeper layers of the sample (Fig. 7).

The northern wall (sample G10, corresponding to G11 sample, fresco “Jacobus Major”) was showing a complex microbial community displaying different groups of organisms entangled in surface layers of the sample. Thinner filaments, possibly actinomycetes (Fig. 8), were attached to the substrate in close contact with mineral particles. As it was already reported, actinomycetes often inhabit art objects (e.g., Giacobini et al., 1988) and in St. Martin actinomycetes were evenly distributed on all surfaces but no correlation with discoloration was observed. Fungal hyphae were dominant here as well (Fig. 9). Faunal elements associated with the biofilms and possibly involved in the spreading of propagules (Gorbushina and Petersen, 2000) were also observed.

Peculiar hyphal cell wall structures (Figs. 4–7) probably connected with the function of dryness survival were frequently observed. The hyphal and sporulation structures were partially collapsed and deformed. This could indicate a status of a still-stand community, which cannot further develop under the prevailing climatic conditions. However, the chains of fungal spores still attached to the material suggest a high propagative and spreading potential of the micromycetes documented. Therefore, any change of the current environmental conditions could trigger a dangerous

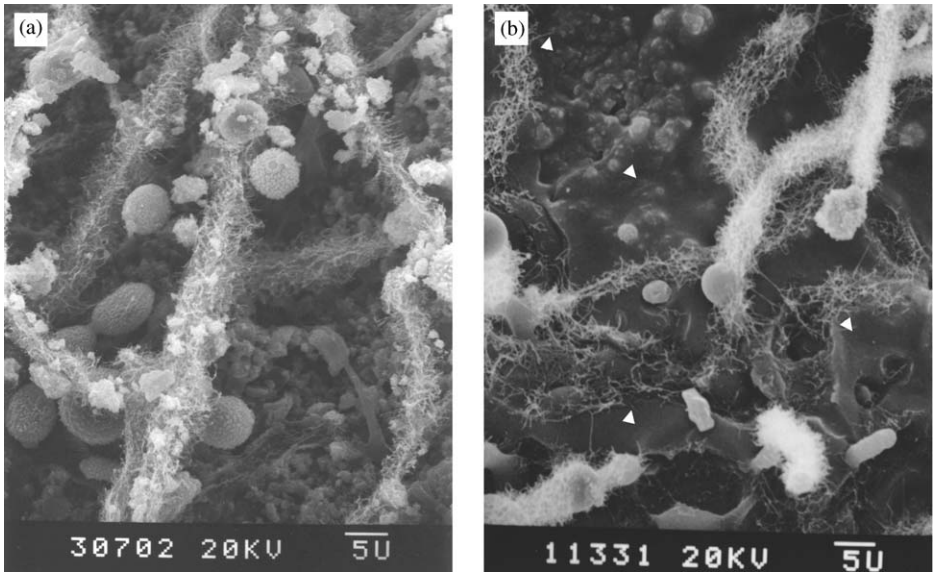

Fig. 4. (a) SEM micrograph of fungal filaments and spores extensively covering the surface of painting layer. Frequently thinner filaments were present that were assigned to growth of actinomycetes; (b) SEM micrograph of fungal growth on the western wall taken by Dr. Karin Petersen in 1991. One can recognize the slimy probably organic material (arrowheads, arthropoda excrements?) on which the first hyphae are starting to develop.

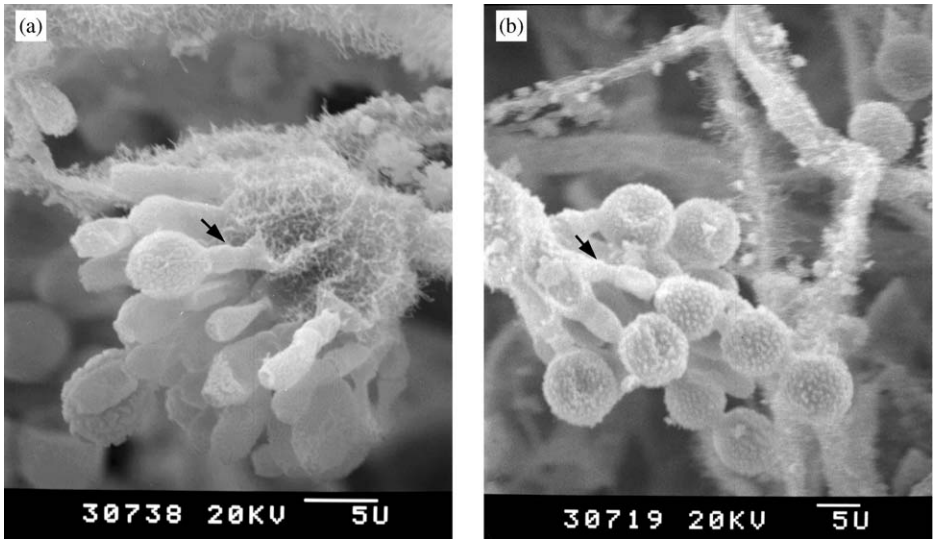

Fig. 5. Fungal growth on the northern (a) and western (b) walls with strikingly similar structure of sporogenous cells (arrows).

new development of the presently inactive but viable biofilm.

Microbial activity of the samples in situ was visualized by staining the sample powder with FDA and propidium iodide. This resulted in no considerable staining of either dead or living cells (results not shown), though SEM clearly demonstrated the presence of microorganisms. This could be explained by the fact that the cells in the present biofilm were dormant and impermeable to the dyes used. The survival structures like spores are predominant and difficult to stain, whereas vegetative cells are not capable of surviving the adverse conditions for longer periods of time and are probably observed only as remnant cell walls. This activity stain failure further supports the hypothesis of a still-standing community waiting for conditions that allow further spreading.

3.2. Isolation and total counts

The total counts of the three different samples used for isolation differed greatly both for bacteria and micromycetes. The samples taken at the north wall (G9 and 11) both showed very little growth on the culture media. On all the culture media used for heterotrophic bacteria, only 3 and 33 colonies

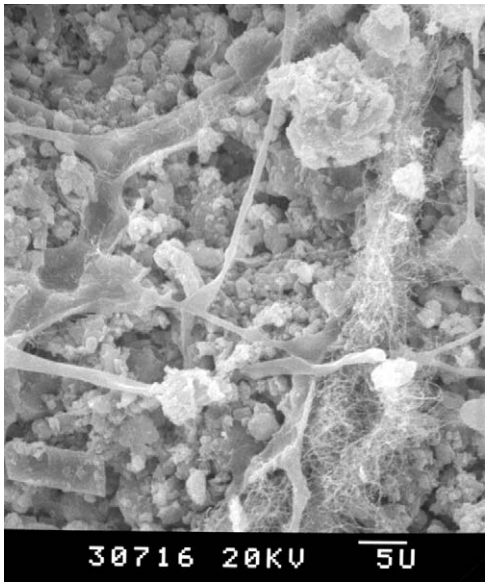

Fig. 6. Development of a close contact between the mural painting and fungal hyphae growth was one of the dominant features. Especially interesting was the observed extracellular matrix of the hyphae, which looks filamentous in its desiccated status.

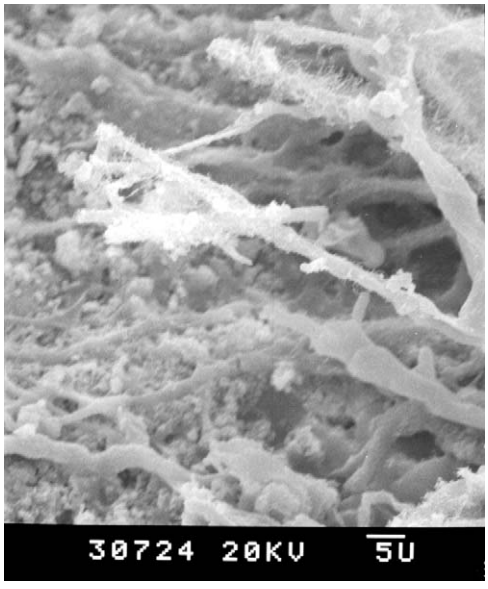

Fig. 7. Fungal growth under the paint layer demonstrates a strong deteriorative potential of the biofilm.

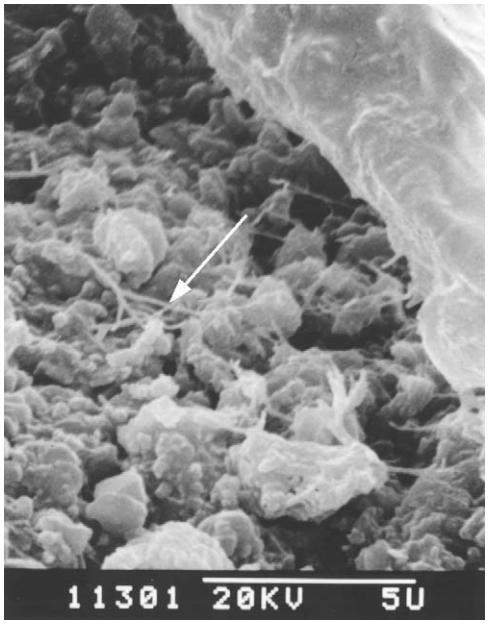

Fig. 8. Thinner possibly actinomycete filaments attached to the substrate are coming into a close contact with mineral particles.

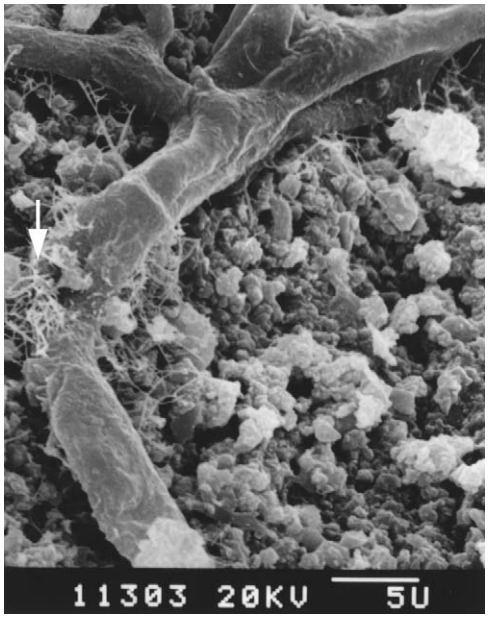

Fig. 9. Fungal hyphae were dominant here as well as on the other wall, with the extracellular matrix (arrow) and in close contact to the painting.

grew, respectively. Dilution plating for fungi resulted in no growth from samples G9 and G11. This was in contrast with the sample taken at the west wall (G4). For this sample the total bacterial counts were in the range of 8.6×10^5–1.4×10^6 colony forming units (cfu) per gram sample for the media without added salt and 3.2×10^4 cfu/g for R2A agar (Difco) with 10% sodium chloride added. A similar difference between the bacterial communities of the samples was observed by Gurtner et al. (2000), where a DNA extraction protocol was used followed by direct amplification (PCR) of a part of the sequence coding for 16S ribosomal RNA in order to conduct denaturing gradient gel electrophoresis (DGGE). A PCR product was obtained directly from sample G4 but not from sample G9 and G11, which further indicates low numbers of bacterial cells in the latter samples. Counts for fungi were equal 3.3×10^4 cfu for G4. Other samples

have not shown any growth in plating experiments. This fact also correlates well with both light and SEM observations.

3.3. Characterization of the bacterial isolates

Of the total amount of 139 bacterial isolates, the different samples G4, G9, and G11 account for 103, 3 and 33 strains, respectively. One hundred and twenty-four strains were grouped together with isolates of other mural paintings sites (according to FAME profiles at 80% Canberra metric similarity, Heyrman et al., 1999). This resulted in 27 different FAME clusters (20, 2 and 10, respectively) and 9 ungrouped strains (2, 1 and 6, respectively). Of the 20 clusters found for the isolates of sample G4, only 4 also contain isolates from sample 9 or 11 (1 and 3, respectively), while 18 of the 20 clusters also contain isolates from one of the other mural painting sites (Carmona and Herberstein). Thus the observed difference in bacterial species composition further confirms the clear separation between the different samples.

The species composition of the different samples is given in Table 1. All analyzed strains of sample G4 were characterized as belonging to the genera *Arthrobacter*, *Bacillus* and *Bacillus*-related genera, all frequently isolated from mural paintings (Weirich, 1989; Karpovich-Tate and Rebrikova, 1990; Altenburger et al., 1996; Ciferri, 1999; Gonzalez et al., 1999). The dominance of bacilli in the samples can be explained by their ability to survive for a long time as spores. Small populations of bacilli present on the mural paintings at a certain period in time can give very high numbers of spores awaiting favorable conditions. Using 16S rDNA sequence analysis, high similarity ($\geqslant$ 99.5%) was found with database entries of *Arthrobacter crystallopoietes*, *Bacillus firmus*, *B. megaterium* and *B. pumilus*. However, for many isolates sequence similarities with the closest related database entries were low enough to differentiate them at the species level ($<$ 97%, Stackebrandt and Goebel, 1994). It can thus be assumed that several of the mural painting isolates belong to novel species. This was already discussed in more detail for the isolates of the mural paintings at the necropolis of Carmona, Seville, Spain (Heyrman and Swings, 2001). Besides the low sequence similarities there was the observation that many strains were closest related to not (yet) validated species of the genera *Bacillus* and *Paenibacillus* (see Table 1), *B.* "*macroides*", *B.* "*pseudomegaterium*", *P.* "*burgondia*" and *P.* "*jamilae*". From the media with added salt (10% NaCl), two clusters of halotolerant strains were isolated, the first was characterized as being closest related to *Gracilibacillus halotolerans* (92.7% sequence similarity) and *B. halodenitrificans* (92.3%), the second showed the highest sequence similarities to *Salibacillus marismortui* (96.5%) and *Virgibacillus proomii* (95.2%). Halotolerant bacteria were previously isolated from other mural paintings (Laiz et al., 2000; Heyrman and Swings, 2001) and can be related with salt accumulation in the walls. From media used for oligotrophic bacteria (Laiz et al., 2002) some isolates characterized by fatty acid analysis, were attributed to other bacterial taxa. Their fatty acid profiles resemble those of *Streptomyces* and sporoactinomycetes members. Also in sample 11, strains characterized by fatty acid analysis as belonging to *Streptomyces* were found.

In a recent study by Gurtner et al. (2000) the bacterial community in sample G4 was also studied by a molecular approach using DGGE analysis. This approach is used to by-pass the cultivation step that is known to select only for a part of the microbial community. Using this technique 15 DGGE bands were obtained. Only one of these bands had the same characterization in 16S rDNA sequence analysis as one of the clusters of cultured strains, namely *A. crystallopoietes* (with 99.8% similarity to the EMBL entry). Other bands were closest to the EMBL database entries of *Rhizobium loti* (97.8%), *Porphyrobacter* sp. (96.7%), *Aquaspirillum delicatum* (97.1%), *Salmonella bongori* (97.5 and 98.3%), *Lysobacter antibioticus* (96.5%), *Geodermatophilus* sp. (98.9%), *Frankia* sp. (98.9%) and *Promicromonospora citrea* (97.9%). For five additional bands no high similarity with any EMBL entry was obtained. A similar discrepancy between cultivation and molecular methods used to characterize the bacterial community on mural paintings was found for the medieval paintings of the chapel in the castle Herberstein (Gurtner et al., 2000). In this study no common results could be found between the cultivation and the molecular approach.

From sample G9, only three strains grew on the presented media. Because of this low number, the possibility that the isolates were mere contaminants was high. Therefore, only the strains, of which the fatty acid profiles grouped together with those of isolates from other sampling points, were characterized further. One of the isolates (LMG 21006) that clustered with strains from sample G11 and from the mural painting sites in Carmona and Herberstein, was further identified by sequencing the 16S rDNA as being closely related to *Staphylococcus hominis* (99.8%). Of sample G11, two additional clusters were assigned to the genus *Staphylococcus* by FAME analysis. One of them was further identified as *S. warneri* (99.9%). Since both species are associated with human skin (Holland and Kearney, 1985; Kloos and Schleifer, 1986) it is possible that they are mere contaminants. However, representatives of both species were found on plates incubated with different samples from different mural paintings sites. In an unpublished report, Jurado et al. (2001) surveyed the microbial populations of seven Sevillian churches. It was found that skin bacteria, and particularly *S. hominis*, *S. saprofiticus*, *S. warneri*, *S. arlettae*, *S. epidermidis* and *S. lugdunensis* were very common in the holy-water fonts. The fonts constituted a pool of skin bacteria, enterobacteria and coliforms and therefore it can be assumed that these bacteria might be present on other surfaces in the indoor church environments. In fact, Saiz-Jimenez

Table 1
Bacterial species composition of the different samples

Identification[a]	LMG-No.[b]	Cluster[c]	No.[d]	Sequence[e]	Closest related database entry[f]
Sample 4					
Arthrobacter crystallopoietes	20239	AJ	4	AJ3 16305 (1477)	*A. crystallopoietes* (X80738, 99.5%)
Arthrobacter sp.	19502^{C}	AK	1	AJ315070 (1485)	*A. citreus* (X80737, 96.3%) *A. aurescens* (X83405, 96.0%)
Gracilibacillus/*Bacillus* sp.	21540	AA	4	AJ491775 (490)	*G. halotolerans* (AF036922, 92.7%) *B. halodenitrificans* (AB021186, 92.3%)
Salibacillus/*Virgibacillus* sp.	19416^{H}	AF	1	AJ276808 (1516)	*S. marismortui* (AJ009793, 96.5%) *V. proomii* (AJ012667, 95.2%)
Bacillus sp.	21004	AG-1	1	AJ316306 (464)	*B.* "*macroides*" (X70312, 99.1%)
	21003	AG-2	3	AJ316307 (480)	*B.* "*macroides*" (X70312, 99.4%)
	21002	AG-3	23	AJ316308 (1503)	*B.* "*macroides*" (AY030319, 99.7%)
Bacillus sp.	20238	AH	3	AJ316309 (1504)	*B.* "*macroides*" (AF157696, 99.6%)
Bacillus megaterium	20240	AO	2	AJ316310 (1504)	*B. megaterium* (D16273, 99.6%)
Bacillus sp.	20246	AS	2	AJ316311 (450)	*B. subtilis* (AB018486, 94.4%) *B. vallismortis* (AB021198, 94.4%)
Bacillus firmus	19496^{C}	AX	4	AJ315064 (1310)	*B. firmus* (D16268, 99.8%)
Bacillus sp.	19497^{C}	AY	6	AJ315065 (1504)	*B. megaterium* (D16273, 99.7%) *B. flexus* (AB021185, 98.9%)
Bacillus pumilus	19499^{C}	BC	7	AJ315066 (1501)	*B. pumilus* (AB020208, 99.8%)
Bacillus sp.	20247	BJ	3	AJ316312 (452)	*B. cereus* (AF176322, 99.8%) *B. anthracis* (AF176321, 99.8%)
Bacillus sp.	20241	—	1	AJ316313 (1502)	*B.* "*pseudomegaterium*" (X77791, 97.4%) *B. methanolicus* (X64465, 95.7%)
Paenibacillus sp.	19508^{H}	AE1	1	AJ315076 (1507)	*P.* "*burgondia*" (AJ011687, 93.4%) *P. popilliae* (AF071860, 93.4%)
	20244	AE2	4	AJ316314 (881)	*P.* "*jamilae*" (AJ271157, 94.3%) *P. popilliae* (AF071859, 94.2%)
Samples 4 and 11					
Bacillus sp.	20248	AI	1+1	AJ316321 (386)	*B.* "*macroides*" (X70312, 99.0%) *B.* "*maroccanus*" (X60626, 97.4%)
Bacillus sp.	19500^{C}	BD	2+1	AJ315067 (1504)	*B. licheniformis* (X68416, 96.2%) *B. sporothermodurans* (U49078, 96.1%)
Paenibacillus sp.	20245	AQ	4+1	AJ316315 (1509)	*P.* "*burgondia*" (AJ011687,94.9%) *P.* "*jamilae*" (AF071860, 94.8%)
Sample 11					
Bacillus sp.	21005	AP	5	AJ316316 (1505)	*B. gibsonii* (X76446, 98.8%)
Bacillus sp.	20243	BA	2	AJ316317 (1430)	*B. cohnii* (AB023412, 95.7%) *B. firmus* (D16268, 95.6%)
Staphylococcus sp.	19417^{H}	BG	1	AJ276810 (1505)	*S. warneri* (L37603, 99.9%) *S. pasteuri* (AB009944, 99.5%)
Nocardioides sp.	20237	BK	1	AJ316318 (1471)	*N. jensenii* (AF005006, 96.1%) *Aeromicrobium erythreum* (AD005021, 95.1%)
Uncharacterized genus	20242	—	1	AJ316319 (1485)	*Terrabacter tumescens* (X83812, 96.9%) *Janibacter limosus* (Y08539, 96.5%)
Samples 11 and 9					
Staphylococcus sp.	21006^{H}	BF	8+1	AJ316320 (1504)	*S. hominis* (X66101, 99.8%) *S. haemolyticus* (X66100, 98.7%)

[a]Characterization of the strains on the basis of 16S rDNA sequence analysis. For some strains a characterization at the species level is assumed because of high sequence similarity with a database entry (EMBL). It must be stated that only DNA–DNA hybridization can confirm this assumption.

[b]Strains were deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie Gent, Ghent, Belgium). Some characterizations given in the table were not obtained by sequence analysis of strains isolated from Greene, but by analysis of strains isolated from the mural paintings of the necropolis of Carmona or the chapel at Herberstein castle of which the fatty acid profiles clustered closely together with those of the Greene strains. Such strains are denoted by x^{C} or x^{H}, respectively.

[c]Fatty acid clusters delineated at 80% Canberra metric similarity (Heyrman et al., 1999). For clusters AG and AE, a representative was chosen from each subcluster obtained when delineating the fatty acid-grouping at 85% Canberra metric similarity to test the exactitude of the clustering.

[d]Number of strains from the Greene samples in each fatty acid cluster.

[e]Accession number and length (amount of base-pairs) of the analyzed sequences.

[f]Closest relatives obtained by comparison with the EMBL database using the FASTA search option (Pearson and Lipman, 1988). Accession numbers and similarity percentages of the closest related database entries are given between brackets. If the difference between the first and the second identification is less then 2% similarity, both identifications are given.

(1981) identified skin bacteria in the mural paintings of La Rabida monastery. Therefore, the identifications of *Staphylococcus* strains are given in Table 1.

The other isolates of sample G11 were assigned to the genera *Bacillus*, *Paenibacillus* and two genera of the actinomycetes. The actinomycetes form a distinct branch of the fatty acid clustering (group II, Heyrman et al., 1999) that only contains six strains from sample G11. The strains of this group were previously attributed to the nocardioform actinomycetes on the basis of their fatty acid profiles (Heyrman et al., 1999). From two of the six strains the 16S rDNA sequence was analyzed. One strain was closest related to *Nocardioides jensenii* (96.1% sequence similarity). Bacteria of the genus *Nocardioides* were recently cultured from cave paintings (Groth et al., 1999). Also DGGE analysis showed the presence of the genus *Nocardioides* in one sample of a medieval mural painting (Gurtner et al., 2000). The other strain was closest related to *Terrabacter tumescens* and *Janibacter limosus* (96.9% and 96.5% sequence similarity, respectively).

3.4. Characterization of the fungal isolates

The total amount of isolated fungal strains equals 32 and accounts mainly for the sample G4 (Table 2). Samples G1, G2 and G9 from which macroscopically visible fungal structures were directly transferred to growth media, have yielded additional isolates. For sampling point G4, the fungal flora obtained after direct transfer of a biofilm fragment to growth media corresponded completely to the results obtained after dilution of the sample followed by plating. A second sampling in May 2000 yielded no additional different isolates. The specific composition of fungal isolates is given in Table 2 and presents various imperfect genera. Fungal genera isolated from the mural painting of the Greene church are frequently mentioned as being typical for mural paintings. Different species of the genera *Acremonium*, *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Penicillium* and *Scopulariopsis* were already mentioned as inhabitants of indoor mural paintings environments (Savulescu and Ionita, 1971; Ionita, 1973; Saiz-Jimenez and Samson, 1981; Sampo and Luppi Mosca, 1989; Rebrikova, 1993; Nugari et al., 1993; Guglieminetti et al., 1994; Berner et al., 1997; Gorbushina and Petersen, 2000). Most species are known to be cave and other indoor environment dwellers, naturally connected with the wall surface environment. Among the isolates high amounts of fungal genera were recorded that are difficult to identify morphologically. In this connection frequently isolated genera of *Acremonium* and microsporic *Fusarium* should be mentioned. This group of strains with unicellular conidia borne on simple phialidic conidiophores is particularly frequent in mural painting environments and constitutes the largest group of the isolates. *Acremonium* and *Fusarium* are common world-wide distributed genera occurring chiefly as soil species or saprophytes. Characterization and identification of strains by BIOLOG had clearly separated between *Acremonium* and morphologically similar microconidial strains of *Fusarium* (Fig. 10). However, one cannot rely on either of the identification methods alone, as type strains of *Acremonium* from a CBS culture collection (*A. charticola* A111, *A. egyptiacum* A112 and *A. rutilum* A114) that were included into the analysis for identification purposes, tend to be extremely different in their nutritional characteristics and do not form a clear group. Moreover, these *Acremonium* strains are seemingly not included into the database of the BIOLOG system. As micromycetes belonging to the genera *Acremonium* and *Fusarium* are frequently isolated from biodeteriorated artifacts, it is definitely useful to broaden the BIOLOG database by molecular and ecophysiological characterization of the numerous existing isolates from mural paintings and other important biodeterioration habitats (for instance in the fungal strain collection of the Geomicrobiology laboratory, Oldenburg University).

The majority of fungal strains was isolated on media with high alkalinity (BRII and PPYG) and was able to grow on media with a difficult carbon source (ECA). Several dark pigmented strains were isolated with the help of PYGV- and

Table 2
Fungal species composition of the different samples

Identification	No.[b]
Sample 1	
Neosartorya fischeri	1
Cladosporium sp.[a]	1
Ulocladium oudesmanii[a]	1
N.I.	1
Sample 2	
Aspergillus sydowii[a]	1
Cladosporium sp.[a]	1
Eupenicillium javanicum	1
Neosartorya sp.[a]	1
Mycelia sterilia[a] melanized	2
Sample 9	
Eupenicillium javanicum[a]	1
Penicillium sp.[a]	3
N.I.	1
Sample 4	
Acremonium sp.	1
Cladosporium sp.	2
Cladosporium sphaerospermum	3
Fusarium sp.	4
Scopulariopsis chartarum	1
Chrysosporium sp.	1
Penicillium sp.	1
Black yeast/*Cladosporium*	1
N.I. hyaline	1
N.I. melanized	2

[a]Isolates obtained by the direct transfer form the wall to the nutrient media.
[b]Number of isolates or when available collection numbers.

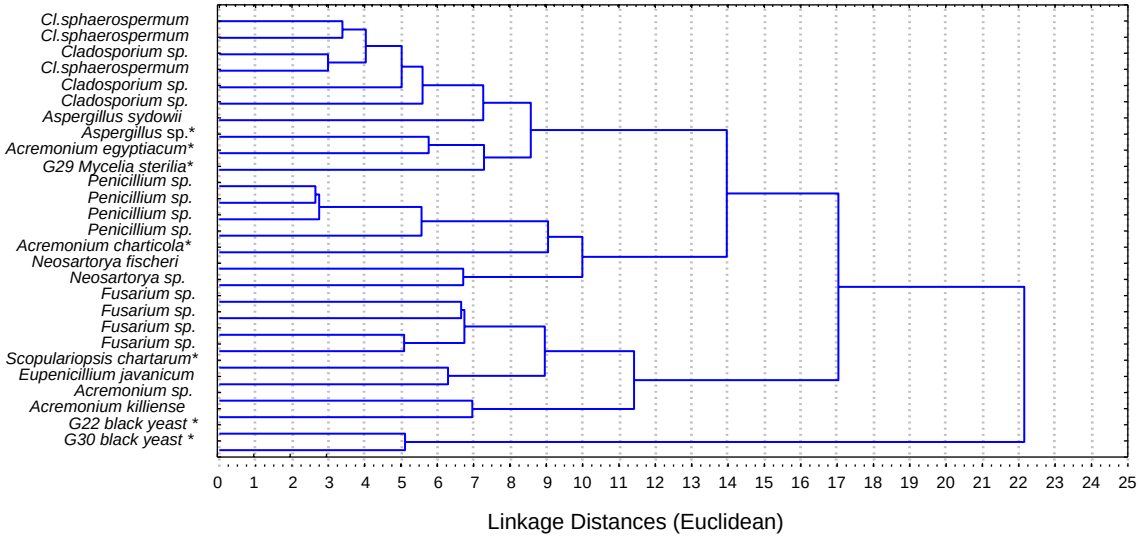

Fig. 10. Greene fungal isolates grouped according to their nutritional requirements (BIOLOG).

ECA-media with a low amount of nutrient and energy sources. Such melanized strains (black yeasts) are known as typical inhabitants of subaerial biofilms in open-air environments (rock surface inhabitants) and are well suited for survival under limiting environmental conditions (Wollenzien et al., 1995; Gorbushina and Krumbein, 2000). These dematiaceous (dark pigmented imperfect fungi) strains were mentioned as being present on wall paintings already some time ago (Tonolo and Giacobini, 1961), but were not widely recognized as typical stress-tolerant mural painting inhabitants. In Greene, two black yeast isolates (G22 and G30) were clearly separated by BIOLOG and form a defined nutritional cluster, but could not be identified even to the genus level. However, melanized fungi are also known as inhabitants of salterns (e.g., Zalar et al., 1999) and other salt and pH stressed environments and thus can represent a small but ecologically important group on mural paintings often highly enriched with salt. Further, a clear cluster of *Cladosporium* strains, undoubtedly defined by morphology, was also separated in its nutritional requirements.

The ambiguity of taxonomical versus nutritional characterization was also reflected in the fact that taxonomically connected teleomorphic and anamorphic isolates from the genera *Neosartorya* and *Aspergillus* remained clearly separated in their nutritional physiology.

The fungal diversity in Greene is thus similar to other mural painting sites. However, the isolation experiments did not correlate well with the microscopical development of the biofilm, since the main biofilm formers observed on the wall by SEM were not among the isolated fungi. It is known that for complex environments, such as mural paintings, we frequently fail to isolate relevant biodeteriorative agents, as we are so far not able to imitate the environmental conditions in a nutrient medium, or to provide the suitable conditions for growth and cultivation.

3.5. Air analysis and environmental parameters

Air spore counts resulted in higher counts of fungal propagules in the vicinity of the western wall (average of 21 cfu in 50l of air). Other collection points have shown less air-borne propagules (3– 5 per 50l). For heterotrophic bacteria the air counts were comparable for the whole church. Strain characterization of the air-borne propagules was determined only for fungi and the genera were identified as *Cladosporium*, *Ulocladium*, *Penicillium,* and *Aspergillus.*

Temperature and humidity measurements have demonstrated a relatively dry indoor climate. The air inside the church had a relative humidity of 52.4 ± 0.5% and a temperature of 18°C. The average values of humidity on the interface with the walls were all between 7.2% and 8.7% (±0.5) for the 11 points measured, and the respective average temperatures were between 17.8 and 18.5°C (±0.1). This demonstrates a low level of humidity throughout the building at that time. Thus it can be stated that the present microclimatic status of the walls is characterized by the absence of significant climatological differences between the northern and the western wall, although the opposite could be expected from the intensity of the biofilm development and the microbial analysis. Therefore, it is assumed that the difference in intensity of microbial growth was related to past environmental situations and a conservation treatment procedure. It is further hypothesized that a local increase of humidity in the western wall during restoration has caused the outburst of a biological activity and has allowed a differentiated biofilm development. Similar rapid development of fungal growths on mural painting was reported for the Chapel of the Castle Herberstein (Styria, Austria). This site was restored in November 1949, using casein-water for consolidation. Only 5 days after this casein application fungal growth was observed (Ochsenfarth, 1998; Piñar et al.,

2001). As the situation seems to be stabilized now, significant differences in the intensity of biofilm development had nivelated during the past decades.

3.6. Biofilm development

In the case of St. Martin church the mural paintings have received no significant organic contributions like casein (Ochsenfarth, personal communication) and the relatively stable climatic conditions at present, especially the dryness of the walls, do not allow the development of a flourishing microbial community. The stimulus for biofilm development probably was the exposure of the murals to the atmosphere after the removal of the white wash in 1977. The restoration procedure may have introduced a considerable amount of humidity (especially the western wall) due to the concrete injections into the wall. The sudden development of the biofilm was first noticed in 1979 and has caused a special attention of the responsible authorities (BIOGEMA Report, 1991; Petersen, personal communication, Drescher Report April 2000). The further spreading of the biofilm was hindered by the relatively stable environmental conditions, possibly only sporadically interrupted by for example condensation humidity caused by gathering of people at the church holidays and the presence of moving arthropoda and their excrements which increase the organic content of the walls and can transfer the spores along the walls (BIOGEMA Report, 1991; Gorbushina and Petersen, 2000). The fact that further spreading of the biofilm was limited is especially underlined by the observation, that later replacements of the painting layer do not show the development of a biofilm (Fig. 3). We therefore conclude that the present viability, spreading patterns, as well as biodiversity of biofilm formers can be used as a record of the habitat history and is helpful in the analysis of a former biodeteriorative situation. A wide representation of spore-formers among bacterial as well as fungal isolates reflects the adaptation possibilities existing for a subaerial system in indoor environment. Under adverse conditions, these microorganisms are forced to respond to them by the formation of structures designed to maintain or restore growth. Environmental conditions favorable for microorganisms have existed for a short period only and have allowed for the development of the biofilm. These observations, combined with SEM analysis, support the hypothesis that the biofilms on the murals in St. Martin church represent a community at a standstill established by an extensive growth period just after the removal of the white wash and wall consolidation treatments in 1977. It is not unusual to observe latent growth structures even decades after the establishment of subaerial biofilms.

Acknowledgements

We thank Monument Care Authorities of Lower Saxony (Dr. Königfeld) and church of Kreiensen-Greene for allowing the sampling and experiments in St. Martin. We acknowledge the financial support of EC through the project “Novel molecular tools for the analysis of unknown microbial communities of mural paintings and their implementation into the conservation/restoration practice” (ENV4-CT98-0705). Further discussions with J. Mertens and technical help of A. Schulte, L. Müller and A. Müller are gratefully acknowledged.

References

Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W., Busse, H.-J., 1996. Classification of bacteria isolated from a medieval wall painting. Journal of Biotechnology 47, 39–52.

Berner, M., Wanner, G., Lubitz, W., 1997. A comparative study of the fungal flora present in medieval wall paintings in the chapel of the castle Herberstein and in the parish church of St. Georgen in Styria, Austria. International Biodeterioration & Biodegradation 40, 53–61.

BIOGEMA Report Krumbein, C., 1991. Untersuchungsbericht zur mikrobiellen Besiedlung der Kirch in Greene (Pilzansammlungen auf den freigelegten Wandmalereien des 16. Jahrhundert), Research and Status Report, 26p.

Bunt, J.S., Rovira, A.S., 1955. Microbiological studies of some sub-Antarctic soils. Journal of Soil Science 6, 119–129.

Ciferri, O., 1999. Microbial degradation of paintings. Applied and Environmental Microbiology 65, 879–885.

Domsch, K.H., Gams, W., Anderson, T.-H., 1980. Compendium of Soil Fungi. Academic Press, London.

Edwards, U., Rogall, T., Blöcker, H., Emde, M., Böttger, E.C., 1989. Isolation and direct compete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research 17, 7843–7853.

Florian, M.-L., 1997. Heritage eaters. Insects and Fungi in Heritage Collections. James & James Ltd, London.

Gee, J.M., Lund, B.M., Metcalf, G., Peel, J.L., 1989. Properties of a new group of alkaliphilic bacteria. Journal of General Microbiology 1127, 9–17.

Giacobini, C., De Cicco, M.A., Tiglie, I., Accardo, G., 1988. Actinomycetes and biodeterioration in the field of fine art. In: Houghton, D.R., Smith, R.N., Eggins, H.O.W. (Eds.), Biodeterioration, Vol. 7. Elsevier Applied Science, New York, pp. 418–423.

Gonzalez, I., Laiz, L., Hermosin, B., Caballero, B., Incerti, C., Saiz-Jimenez, C., 1999. Bacteria isolated from rock art paintings: the case of Atlanterra shelter (south Spain). Journal of Microbiological Methods 36, 123–127.

Gorbushina, A.A., Krumbein, W.E., 2000. Subaerial microbial mats and their effects on soil and rock. In: Riding, R.F., Awramik, S.M. (Eds.), Microbial Sediments. Springer, Berlin, pp. 161–170.

Gorbushina, A.A., Petersen, K., 2000. Distribution of micro-organisms on ancient wall paintings as related to associated faunal elements. International Biodeterioration & Biodegradation 46, 277–284.

Gramann, C., 1998. Versuche und Eroerterungen zur antimikrobiellen Ausrüstung von Caseinrezepturen in der Wandmalereirestaurierung. Facharbeit zum Diplom, FH Hildesheim/Holzminden, 57p.

Groth, I., Vettermann, R., Schuetze, B., Schumann, P., Saiz-Jimenez, C., 1999. Actinomycetes in Karstic caves of northern Spain (Altamira and Tito Bustillo). Journal of Microbiological Methods 36, 115–122.

Guglieminetti, M., De Guili Morghen, C., Radaelli, A., Bistoni, F., Carruba, G., Spera, G., Caretta, G., 1994. Mycological and ultrastructural studies to evaluate biodeterioration of mural paintings. Detection of fungi and mites in frescos of the monastery of St. Damian in Assisi. International Biodeterioration & Biodegradation 34, 269–283.

Gurtner, C., Heyrman, J., Piñar, G., Lubitz, W., Swings, J., Rölleke, S., 2000. Comparative analyzes of the bacterial diversity on two different biodeteriorated wall paintings by DGGE and 16S rDNA sequence analysis. International Biodeterioration & Biodegradation 46, 229–239.

Heyrman, J., Swings, J., 2001. 16S rDNA sequence analysis of bacterial isolates from biodeteriorated mural paintings in the Servilia tomb (Necropolis of Carmona, Seville, Spain). Systematic and Applied Microbiology 24, 417–422.

Heyrman, J., Mergaert, J., Denys, R., Swings, J., 1999. The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by microorganisms. FEMS Microbiology Letters 181, 55–62.

Holland, K.T., Kearney, J.N., 1985. Microbiology of skin. In: Skerrow, D., Skerrow, C. (Eds.), Methods in Skin Research. Wiley, Sussex, England, pp. 433–473.

Ionita, I., 1973. Contributions to the study of the biodeterioration of the work of art and of historic monuments. II. Species of fungi involved in the deterioration of mural paintings from the monasteries of Moldavia. Revue Roumaine de Biologie, Serie Botanique 18, 179–189.

Jurado, V., Ortiz, A., Gonzalez-delValle, M., Laiz, L., Saiz-Jimenez, C., 2001. Estudio de las poblaciones bacterianas presents en las pilas de agua bendita de siete templos de la ciudad de Sevilla. Internal Report IRNAS, 27p.

Karpovich-Tate, N., Rebrikova, N.L., 1990. Microbial communities on damaged frescoes and building materials in the cathedral of the Nativity of the Virgin in the Pafnutii-Borovskii monastery, Russia. International Biodeterioration & Biodegradation 27, 281–296.

King Jr., A.D., Hocking, A.D., Pitts, J.I., 1979. Dichlorane Rose Bengal medium for enumeration and isolation of microorganisms from food. Applied and Environmental Microbiology 37, 959–964.

Kloos, W.E., Schleifer, K.H., 1986. Staphylococcus. In: Sneath, P.H., Mair, N., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins, Baltimore, MD, pp. 1013–1035.

Laiz, L., Recio, D., Hermosin, B., Saiz-Jimenez, C., 2000. Microbial communities in salt efflorescences. In: Ciferri, O., Tiano, P., Mastromei, G. (Eds.), Of Microbes and Art—The Role of Microbial Communities in the Degradation and Protection of Cultural Heritage. Kluwer Academic, Dordrecht, Plenum Publishers, New York, pp. 77–88.

Laiz, L., Hermosin, B., Caballero, B., Saiz-Jimenez, C., 2002. Facultatively oligotrophic bacteria in Roman mural paintings. In: Galan, E., Zezza, F. (Eds.), Protection and Conservation of the Cultural Heritage of the Mediterranean Cities. Balkema, Swets and Zeitlinger Publishers, Lisse, pp. 173–178.

Nugari, M.P., Realini, M., Roccardi, A., 1993. Contamination of mural paintings by indoor airborne fungal spores. Aerobiologia 9, 131–139.

Ochsenfarth, 1998. Brief report on the analysis of the inventory of paintings and of the sampling of microbial material in the Catherine Chapel of Herberstein Castle. Internal Report for Microcore Project. Lüeck, 24p.

Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. Proceedings of the National Academy of Sciences of the United States of America 85, 2444–2448.

Piñar, G., Saiz-Jimenez, C., Shabereiter-Gurtner, C., Blanco-Varela, M.T., Lubitz, W., Rölleke, S., 2001. Archaeal communities in two disparate deteriorated ancient wall paintings: detection, identification and temporal monitoring by DGGE. FEMS Microbiology Ecology 37 (1), 45–54.

Pitcher, D.G., Saunders, N.A., Owen, R.J., 1989. Rapid extraction of bacterial genomic DNA with guanidium thio-cyanate. Letters in Applied Microbiology 8, 151–156.

Rebrikova, N.L., 1993. Micromycetes taking part in deterioration of old Russian wall paintings. In: Garg, K.L., Garg, N., Mukerji, K.G. (Eds.), Recent Advances in Biodeterioration and Biodegradation, Vol. I, Naya Prokash, Calcutta, pp. 205–232.

Saiz-Jimenez, C., 1981. Los murales de Sta M[a] de la Rábida: causas del deterioro. Mundo Científico 18, 1007–1011.

Saiz-Jimenez, C., Samson, R.A., 1981. Microorganisms and environmental pollution as deteriorative agents of the frescoes of the monastery of Santa Maria de la Rabida, Huelva, Spain. Proceedings of Sixth Triennial Meeting of ICOM Committee for Conservation, Ottawa, pp. 81/15/5-1–14.

Sampo, S., Luppi Mosca, A.M., 1989. A study of the fungi occurring on 15th century frescoes in Florence, Italy. International Biodeterioration & Biodegradation 25, 343–353.

Savulescu, A., Ionita, I., 1971. Contribution to the study of the biodeterioration of the works of art and historic monuments. I. Species of fungi isolated from frescoes. Revue Roumaine de Biologie. Serie Botanique 16, 201–206.

Stackebrandt, E., Goebel, B.M., 1994. Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology 44, 846–849.

Staley, J.T., 1968. Prosthecomicrobium and Ancalomicrobium — new prosthecate freshwater bacteria. Journal of Bacteriology 95 (5), 1921–1942.

Tonolo, A., Giacobini, C., 1961. Microbiological changes of frescoes. In: Thomson, G. (Ed.), Recent Advances in Conservation. Butterworths, London, pp. 62–64.

Weirich, G., 1989. Untersuchungen über Mikroorganismen von Wandmalereien. Material und Organismen 24, 139–159.

Wollenzien, U., de Hoog, G.S., Krumbein, W.E., Urzì, C., 1995. On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. Science of the Total Environment 167, 287–294.

Zalar, P., de Hoog, G.S., Gunde-Cimerman, N., 1999. Ecology of halotolerant dothideaceous black yeasts. Studies in Mycology 43, 38–48.