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A multi-proxy approach to evaluate biocidal treatments on biodeteriorated majolica glazed tiles

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Summary

The Fishing House located on the grounds of the Marquis of Pombal Palace, Oeiras, Portugal, was built in the 18th century. During this epoch, Portuguese gardens, such as the one surrounding the Fishing House, were commonly ornamented with glazed wall tile claddings. Currently, some of these outdoor tile panels are covered with dark coloured biofilms, contributing to undesirable aesthetic changes and eventually inducing chemical and physical damage to the tile surfaces. Phylogenetic analyses revealed that the investigated biofilms are mainly composed of green algae, cyanobacteria and dematiaceous fungi. With the aim of mitigating biodeterioration, four different biocides (TiO_2 nanoparticles, Biotin® T, Preventol® RI 80 and Albilex Biostat®) were applied *in situ* to the glazed wall tiles. Their efficacy was monitored by visual examination, epifluorescence microscopy and DNA-based analysis. Significant changes in the microbial community composition were observed 4 months after treatment with Preventol® RI 80 and Biotin® T. Although the original community was inactivated after these treatments, an early stage of re-colonization was detected 6 months after the biocide application. TiO_2 nanoparticles showed promising results due to their self-cleaning effect, causing the detachment of the biofilm from the tile surface, which remained clean 6 and even 24 months after biocide application.

Keywords: Glazed tiles, biofilms; biocides; DGGE; TiO_2

Introduction

The Marquis of Pombal Palace (*Palácio Marquês de Pombal* in Portuguese) was built during the 18th century in Oeiras, which is located in the western part of what is now the Lisbon Metropolitan Area (Portugal). This Portuguese baroque manor house was

connected to an estate. A cottage surrounded by a garden is located in the middle of the estate. This cottage has an artificial fish pond that was used for ludic activities such as fishing, giving it the name of the Fishing House. The garden surrounding the Fishing House is of neoclassical style decorated with a fountain, a cascade, terraces and stairs with several tile panels adorning the walls. During the 18th century, glazed tiles were commonly used as decorative elements in Portuguese gardens, ornamenting fountains, stairs, benches and walls.

Currently, the Marquis of Pombal Palace and estate are separated, as the estate is used as an agronomical station. Unfortunately, the Fishing House and the surrounding garden were unkempt and are now extremely deteriorated. The glazed wall tiles are covered with dark coloured biofilms, contributing to undesirable aesthetic changes and eventually inducing chemical and physical damage to the tile surfaces.

Little is known about the biodeterioration of glazed ceramic tiles, compared to that of other inorganic building materials (Coutinho *et al.*, 2015). Only recently has this topic attracted interest, and several studies have been published (Oliveira *et al.*, 2001; Watanabe *et al.*, 2006, 2009; Giacomucci *et al.*, 2011; Coutinho *et al.*, 2013). The assessment of biodeterioration demands the characterization of the substratum and the organisms implicated (Alakomi *et al.*, 2006). Such information allows the understanding of the role played by microorganisms in biodeterioration processes and is essential to the design and selection of appropriate preventive and eradication methods. An efficient mitigation strategy should inactivate the organisms and prevent their re-growth for an acceptable period of time, causing no alteration to the substratum (Young *et al.*, 2008).

The eradication of microorganisms from inorganic building materials usually involves mechanical procedures and/or the application of biocides (chemical methods).

However, both methods have a number of drawbacks. Mechanical removal of biofilms is tedious and ineffective, and can even spread the microorganisms or cause staining on the surface (Fig. 1c), resulting in a more severe colonization (Jurado *et al.*, 2014). Moreover, if tiles are in a fragile conservation condition with areas in detachment, the mechanical removal can enhance the risk of partial glaze loss.

Commercial biocides, like Biotin® T and Preventol® RI 80, have been widely applied on inorganic substrates from cultural heritage materials, such as stone and mortars (Ascaso *et al.*, 2002; Fonseca *et al.*, 2010; De los Ríos *et al.*, 2012). However, studies on their long-term efficacy are scarce, and the potential re-colonization has not been monitored after biocide application for any of them. The fact that biocides may not promote a long-term protection has created some scepticism, because microbial communities may develop resistance mechanisms (Morton *et al.*, 1998; Bastian *et al.*, 2009). Moreover, the original microbial community may be replaced by microorganisms previously absent from the substratum due to the presence of biocide residues, leading to new microbial outbreaks (Bastian *et al.*, 2009; Martin-Sanchez *et al.*, 2012).

Regarding the removal of biofilms from glazed tiles and ceramic materials, there are no specific guidelines. Some authors reported the removal of biofilms from ceramics using different procedures, such as chemical, thermal (heating) and gamma-radiation treatments (John, 1988; Oliveira *et al.*, 2001; Silva *et al.*, 2011; Mandal and Rath, 2013). However, some methods produced undesirable changes on the substratum or had no long-term effect (Giacomucci *et al.*, 2011; Silva *et al.*, 2011; Mandal and Rath, 2013).

In this research study, majolica glazed tiles from the Fishing House were characterized, and the associated microbial communities were identified using both

culture and molecular biology methods. Afterwards, three different commercial biocides (Biotin® T, Preventol® RI 80 and Albilex Biostat®), and a heterogeneous photocatalyst based on nanocrystalline anatase titanium dioxide (TiO_2) nanoparticles were tested *in situ*. Their efficacy was monitored after 4 and 6 months of application by photographic recording, epifluorescence microscopy and denaturing gradient gel electrophoresis (DGGE). Finally, the effect of each treatment on the substratum was assessed by scanning electron microscopy (SEM).

Results

Macroscopic and microscopic observations

The glazed wall tiles from the Fishing House showed biodeterioration damage caused by biofilm development on their surfaces (Fig. 1). Microbial colonization developed profusely on these tiles as brownish biofilm covering the panels, causing severe aesthetic and eventually physical and chemical biodeterioration (Fig. 1a,b).

Fig. 1 Near here

Under the stereomicroscope, the colonized tile fragments showed a dense green to brownish-coloured coating on the glazed surface. Disk-shaped thalli were also observed in some areas of the colonized surface and chasmoendolithic growth was evident in glaze fissures (Fig. S1). It is noteworthy the extensive biological colonization found on the ceramic body as well as beneath the glaze layer, particularly by photoautotrophic microorganisms (Fig. S1).

Light microscope images of fresh biofilm samples collected from the surface of the glazed tiles allowed the identification of the green alga *Phycopeltis arundinacea* (Fig. 2). This microalga exhibits a green and orange-brown colouration, and disk-shaped thalli with lobed margins (Fig. 2a). The *Phycopeltis arundinacea* cells were

found to be surrounded by brown hyphae of dematiaceous fungi (Fig 2b). Although a close interaction between the melanised fungi and algae was observed, no lichens or lichenization process were identified in the biofilm samples.

Fig. 2 Near here

The SEM analysis of glaze fragments, directly mounted on sample stubs and sputter-coated with gold, corroborated the copious microbial colonization observed by stereomicroscopy (Fig. 3). Algal thalli with their characteristic margins and fungal hyphae were evident (Fig. 3a, b and c). Figure 3d,e shows the microbe-glaze interface. The adhesion of microorganisms to the glaze by extracellular polymeric substances (EPS) is clearly observed. Fragments of the broken glaze that remained attached to the thallus can also be observed (Fig. 3d). The unilayered thallus of *Phycopeltis arundinacea* strongly adherent to the glaze by EPS is also evident in Figure 3e.

Fig. 3 Near here

Fungal isolation and identification by molecular biology techniques

Eight fungal strains (isolates CP1 to CP8) were isolated from the brownish biofilm. The identification of these isolates was performed by DNA-based analysis, which revealed three different fungal taxa belonging to the Capnodiales order of the Ascomycota phylum, with similarities between 94 and 99% (Table 1).

These taxa belong to the recently described *Neodevriesiaceae* family (Quaedvlieg *et al*., 2014), which resulted from a re-distribution of genera within the *Teratosphaeriaceae* family. Based on molecular analysis, these fungi were identified as *Neodevriesiaceae* sp. 1 (closely related to *Neodevriesia xanthorrhoeae*), *Devriesia modesta* and *Neodevriesiaceae* sp. 2.

154 ***Efficacy of the biocidal treatments applied on glazed wall tiles***

155 Four biocides were applied *in situ* on glazed wall tiles depicting severe colonization: i)
156 TiO_2 nanoparticles; ii) Preventol® RI 80 (hereinafter Preventol); iii) Biotin®T
157 (hereinafter Biotin), and iv) Albilex Biostat® (hereinafter Albilex).

158

159 *Visual inspections*

160 Macroscopic observations revealed that the biocide treatments induced biofilm changes
161 in terms of colour, morphology and texture, with the exception of Albilex (Fig. 4).

162

163 Fig. 4 Near here

164 After the application of the TiO_2 photocatalytic biocide, variation of the biofilm
165 colour and density was not evident. It was, however, noticeable a significant decrease in
166 the biofilm coverage area, as well as in the adhesion of the biofilm to the substratum
167 (Fig. 4). Figure 5 shows the biofilm flaking off from the tile surface 6 months after the
168 application of TiO_2 nanoparticles.

169 Fig. 5 Near here

170

171 Biotin induced a slight colour variation of the biofilm, but the most noticeable
172 effect was the loss of density at the end of the experiment (Fig. 4). Preventol caused the
173 most significant changes to the biofilm, altering its colour, density and thickness (Fig.
174 4). These biocide effects were progressive after the treatment, but became significantly
175 evident 4 months after the biocide application (Fig. 4). Only the Biotin and Preventol
176 treatments allowed the visualization of the tile motifs, but the degraded biofilm was still
177 present on the glazed tiles.

178

Epifluorescence microscopy

The effect of the four biocide treatments on the photosynthetic community was analysed by epifluorescence microscopy, after 4 and 6 months of the biocide application (Fig. 6).

After 4 months, an apparent decrease in the red light emission was observed for all treatments, which indicates the degradation of chlorophyll *a* (Chl*a*), except for the biofilm treated with Albilex (Fig. 6).

Fig. 6 Near here

The non-treated biofilm (control) and that treated with Albilex showed similar Chl*a* fluorescence intensities (Fig. 6). The absence of Chl*a* fluorescence in the samples treated with Biotin and Preventol was particularly evident, as well as the disruption of the algal structures (Fig. 6). Regarding the samples treated with the TiO_2 biocide, punctual inactivation was observed in small areas displaying white-blue fluorescence which indicates Chl*a* degradation (Fig. 6). After 4 months, Preventol and Biotin were shown to be the most efficient treatments for the inactivation of photosynthetic cells, reducing the active Chl*a* concentration as no red fluorescence was observed. In contrast, Albilex seemed to be ineffective as Chl*a* fluorescence was amply observed along the sample.

The comparison of the data obtained by epifluorescence 4 and 6 months after the treatments revealed that the fluorescence of the non-treated biofilm (control) was preserved (Fig. 6). After 6 months, the biofilm samples from the Biotin- and Preventol-treated areas showed small spots of red fluorescence, whereas the TiO_2- and Albilex-treated biofilm samples depicted red fluorescence similar to the non-treated biofilm (Fig. 6).

Monitoring of microbial communities by DGGE

DGGE was used to monitor the changes in the microbial community composition of the Fishing House glazed tiles before and 4 months after the application of the biocides. Figure 7 shows the DGGE profiles derived from the cyanobacterial 16S rDNA (Fig. 7a), fungal ITS (Fig. 7b) and eukaryotic 18S rDNA (Fig. 7c).

Fig. 7 Near here

Regarding cyanobacteria, the DGGE profiles of the non-treated biofilm samples were identical in composition during the 4 months of the experiment (Fig. 7a, lanes C0 and C4). The biofilm samples treated with TiO_2 also showed similar DGGE pattern to the non-treated biofilms (Fig. 7a, lane T4). It is noteworthy that no amplification was obtained for cyanobacterial 16S rDNA in the sample collected from the area treated with Biotin (B4). A visible change in the microbial community composition was observed for the samples treated with Preventol (Fig. 7a, lane P4). The sample from the Albilex-treated biofilm (A4) showed a DGGE profile similar to that of the non-treated samples (C0 and C4), depicting only one DGGE band in common with sample P4 (Fig. 7a, lane A4).

The fungal community profiles of the non-treated biofilms (Fig. 7b, lanes C0 and C4) showed a stable band pattern during the 4 months of the experiment. In fact, the observed bands were coincident with the DGGE pattern of the viable fungal species described in Table 1 (Fig. 7b): *Devriesia modesta* (band 1), *Neodevriesiaceae* sp. 2 (band 2) and *Neodevriesiaceae* sp. 1 closely related to *Neodevriesia xanthorrhoeae* (band 3). TiO_2 and Albilex seemed not to cause changes in the fungal community composition, when comparing their DGGE patterns with the non-treated biofilms (Fig. 7b, lanes T4 and A4). On the contrary, a noticeable alteration occurred after the application of Biotin or Preventol (Fig. 7b, lanes B4 and P4). The changes in the DGGE

pattern were more drastic in the samples treated with Preventol than in those treated with Biotin. Faded bands (numbers 1 and 3) positioned equally to the dominant bands of the non-treated samples were observed in the Biotin-treated sample. These bands corresponded to the fungi *Devriesia modesta* and *Neodevriesiaceae* sp. 1, respectively (Fig. 7b lane C0).

Similarly, the eukaryotic 18S rDNA gene DGGE profiles did not show any variation in the banding patterns of the non-treated samples, and for the biofilm samples treated with TiO_2 or Albilex (Fig. 7c, lanes C0, C4, T4, A4). DGGE profiles of the biofilm treated with Biotin (Fig. 7c, lane T4) and Preventol (Fig. 8c, lane P4) showed some changes compared to those of the non-treated biofilm samples (Fig. 7c). Again, the DGGE banding pattern of the Biotin-treated sample showed faded bands corresponding to some of the dominant bands of the non-treated samples. The changes in the DGGE pattern were more drastic in samples treated with Preventol.

Phylogenetic identification of microbial communities before and after* in situ *application of biocide

DNA libraries were constructed for the non-treated biofilm and the Preventol-treated biofilm sample. This latter was selected according to DGGE analysis, as this treatment showed the greatest variation in the DGGE banding pattern.

DNA-based analyses of the non-treated biofilm revealed a microbial community mainly composed of photosynthetic microorganisms (chlorophyta and cyanobacteria) and fungi (Tables 2-4, sample C0). A total of 85 clones were retrieved for the non-treated sample (C0), with 7 taxa identified (Tables 2-4). For the biofilm treated with Preventol, 4 months after its application, a total of 60 clones were obtained. DNA-based analyses showed cyanobacteria and eukaryotes as the main components (Tables 2-4,

sample P4). After this biocide treatment, a higher biodiversity was observed, with 20 taxa belonging to Chlorophyta, Ascomycota, Basidiomycota, Cyanobacteria, Verrucomicrobia and Proteobacteria. The similarity of the sequences retrieved varied between 94 and 99% for cyanobacteria, 98 and 100% for fungi, and 96 and 99% for eukaryotes with their closest homologue sequences from GenBank (Tables 2-4).

In the cyanobacterial 16S rDNA analysis, 37 clones were obtained from the non-treated biofilm (C0), showing similarities of 99% with their closest homologue sequences and between 91 and 97% with the closest cultured sequences (Table 2, sample C0). Among them, Chlorophyta chloroplasts represented 97% of the obtained 16S rDNA sequences using primers specific for cyanobacteria, and only 3% of the sequences were related to cyanobacteria. These sequences were affiliated with uncultured eukaryote plastids (99% similarity) and reached a low similarity with its nearest cultured relative *Oocystis solitaria* (91% similarity). The identified uncultured cyanobacteria belonged to the *Iphinoe* genus and the closest cultured relative was *Iphinoe spelaeobios* (Table 2).

The cyanobacterial sequences obtained from the Preventol-treated sample showed higher biodiversity than the non-treated sample did and were composed of Cyanobacteria, Verrucomicrobia and Proteobacteria (Table 2, sample P4). Cyanobacteria were the dominant phylum among the prokaryotic members of the treated biofilm (60%). The nearest cultured relatives were *Tolypothrix distorta* and *Anabaena sphaerica* representing each 30% of the total clones. Verrucomicrobia and Proteobacteria were represented by the species *Luteolibacter pohnpeiensis* (10%) and *Rhizobium* sp. (10%), respectively (Table 2). Chlorophyta chloroplasts were also obtained using primers specific for cyanobacteria. *Oocystis solitaria* and *Coccomyxa*

subellipsoidea were the closest cultured relatives, representing each 10% of the total clones (Table 2).

In the Ascomycota present on the non-treated biofilm covering the glazed wall tiles, the same three isolated fungi belonging to the *Neodevriesiaceae* family were identified: *Neodevriesiaceae* sp. 2 (61% of clones), *Neodevriesiaceae* sp. 1 related to *N. xanthorrhoeae* (35%) and *D. modesta* (4%) (Tables 1 and 3).

The ITS sequences obtained from the Preventol-treated biofilm belonged to Ascomycota and Basidiomycota (Table 3). The phylum Ascomycota was dominant, representing 80% of the total clones. Most of the sequences retrieved by this analysis belonged to the species *Aureobasidium pullulans* (68%). Two other members of the same phylum *Chalara* sp. (11%) and *Phoma pereupyrena* (5%), were also identified (Table 3). Basidiomycota was represented by three species of the same genus: *Cryptococcus carnescens* (11%), *C. dimennae* (5%) and *C. foliicola* (5%) (Table 3).

Among the eukaryotes obtained from the 18S rDNA analysis of the non-treated biofilm, Chlorophyta represented 96% of the retrieved clones, comprising solely the genus *Phycopeltis*. A minor proportion of the sequences (4%) belonged to an unclassified Ascomycota from the Capnodiales order (Table 4).

The eukaryotic analysis of the Preventol-treated biofilm revealed organisms belonging to Chlorophyta, Ascomycota and Basidiomycota. The Chlorophyta *Phycopeltis* sp. was also present in the Preventol-treated biofilm, but this green alga represented only 7% of the clones retrieved from the treated sample, compared to the 96% obtained from the non-treated biofilm (Table 4). The highest proportion of clones obtained from the treated biofilm belonged to the phyla Ascomycota and Basidiomycota, representing 33 and 60% of total clones, respectively. The species *Aureobasidium pullulans* (30%) represented a major part of the Ascomycota clones,

while the remaining 3% were closer to an uncultured Ascomycota clone (Table 4). Basidiomycota were represented by three species of the same genus*: Cryptococcus* sp., *C. magnus* and *C. psychrotolerans* (Table 4). Two other Basidiomycota members, *Kondoa malvinella* and *Trichosporon mucoides*, were also related to the sequences retrieved from the Preventol treated-biofilm, each representing 3% of the analysed sequences (Table 4).

Figure 8 illustrates the phylogenetic study of the fungal community present on the biofilms before treatment and 4 months after the Preventol biocide application. All isolated fungal strains and representative clones of this study were classified into three different clades (Fig. 8). One of them, supported by a 100% bootstrap value, was identified as *Devriesia modesta*. The other two taxa (*Neodevriesiaceae* spp. 1 and 2), the most abundant in the biofilm, were independent clades supported by high bootstrap values (72–99%). Four months after the application of Preventol, the formerly dominant species of the *Neodevriesiaceae* family were no longer detected. The exposure to the biocide created a shift in the phylogenetic community structure. A higher biodiversity of several lineages of fast growing Ascomycota and Basidiomycota were retrieved from the Preventol-treated biofilm (Table 3, Fig. 8).

Morphological, chemical and mineralogical characterization of glazed wall tiles

The white glaze of the majolica tiles cladding the handrails of a stair in the Fishing House were analysed in terms of the microstructure, mineralogical and chemical composition. Variable pressure scanning electron microscope (VP-SEM) images of a glaze cross-section revealed a heterogeneous microstructure with inclusions of different composition, shape and size (Fig. 9). Element mapping by energy dispersive X-ray spectroscopy (EDS) showed the distribution of these inclusions, which were rich in

silica, potassium and tin (Fig. 9a-c). EDS microanalysis of the white glaze revealed a silica lead glaze opacified with tin oxide crystals (Fig. 9d).

Fig. 9 Near here

The mineralogical composition of the inclusions was then determined by μ-Raman spectroscopy. The characteristics peaks of quartz, cassiterite and potassium feldspar were identified in the Raman spectra (Fig. S2).

The average chemical compositions of four white glaze samples obtained by micro-particle induced X-ray emission (μ-PIXE) are summarized in Table 5. According to the contents obtained, the glaze can be considered an alkali-lead silicate, with SiO_2 being the major component (58 wt. %). Lead and potash were the main fluxing agents, representing 22 and 5.7 wt. % of the glaze, respectively. In majolica glazes, the main opacifier is tin oxide, with a content of approximately 4.9 wt. %.

The eventual changes induced in the glaze substratum by the applied treatments were monitored by means of VP-SEM. Figure 10 depicts the glaze surfaces, with no substantial changes in the treated and untreated colonized glazes. For all analysed samples, fissures, pitting and accumulation of lead-rich corrosion products were observed (Fig. 10). Superficial deposits, were detected both on the untreated and treated glazed tiles (Fig. 10).

Fig. 10 Near here

Tile status after 2 years

Macroscopic inspection was performed two years after the application of the biocides (Fig. S3). In the TiO_2-treated area, the biofilm detached from most of the tested tile surface, unveiling the tile motifs and high brightness of the glaze. In the Biotin and Preventol-treated areas, a thin layer of brownish biofilm still remained on the glazed

surface, giving it a matte appearance. Finally, the Albilex-treated area showed no visual changes of the biofilm in comparison with the surrounding areas where no biocide was applied (Fig. S3).

Discussion

Microbial community growth on the glazed tiles

The brownish biofilm developing on the glazed tiles from the Fishing House was mainly composed of chlorophyta, together with fungi belonging to the *Neodevriesiaceae* family, a cyanobacterium from the *Iphinoe* genus and a few bacterial species (Tables 1-4). Light microscopy observations of fresh biofilm samples revealed the presence of the alga *Phycopeltis arundinacea*, which was corroborated by DNA-based analysis (Table 4). This subaerial green alga is mainly epiphytic on the leaves of perennial terrestrial plants (John, 2003; Rindi and Guiry, 2002; Rindi *et al.*, 2004). To our knowledge, it has not been reported on cultural heritage assets or on any inorganic building material. In contrast, the chlorophyta *Oocystis solitaria* was previously identified on glazed tiles from Pena National Palace in Sintra, Portugal, but with very low similarity to the mentioned sequence (Coutinho *et al.*, 2013)(Table 2). The cyanobacterium *Iphinoe* sp., identified on the non-treated biofilm, was reported in Greek and Spanish caves (Lamprinou *et al*., 2011).

For the fungal community, DNA-based analysis and culture-dependent methods showed similar results. Ascomycota members of the Capnodiales order, especially from the *Neodevriesiaceae* family, were identified in the non-treated biofilm (Table 1, 3 and 4). Slow-growing species of rock-inhabiting fungi belonging to Capnodiales are resistant to harsh climatic conditions due to their strongly melanized dark-coloured cell walls (Ruibal *et al.*, 2008; Egidi *et al.*, 2014). The *Neodevriesiaceae* family was the

most common fungal taxon detected in the Fishing House biofilms. This novel family was recently described grouping members of the genera *Devriesia*, *Neodevriesia*, *Teratosphaeria* and *Tripospermum*. The first identified members of *Devriesia* were heat-resistant fungi isolated from heat-treated soils (Seifert *et al.*, 2004). Indeed, glazed surfaces exposed to direct sunlight during hot seasons can achieve relatively high surface temperatures. A new species of this genus, *Devriesia imbrexigena*, was isolated from a similar substratum, the silica-lead glaze from the wall tiles of the Pena National Palace in Sintra, Portugal (Crous *et al.*, 2012). The two most abundant fungi in the biofilm from the Fishing House glazed tiles could represent unknown species of the *Neodevriesiaceae* family, considering their low similarity percentages with the currently described species.

Although the biodeterioration damages caused by these fungi on ceramic glazed substrata are still unknown, several authors have associated these fungi with aesthetic biodeterioration on stone (Sert *et al.,* 2007, 2010; Cutler *et al.,* 2013*;* Egidi *et al.*, 2014). Most of the retrieved sequences from the non-treated biofilm showed high similarity with microorganisms found on stone cultural heritage assets (e.g. Roman Necropolis of Carmona, Iranian archaeological heritage, Pena National Palace tiles) or soil (Bonazza *et al.*, 2007; Benavente *et al.*, 2011; Coutinho *et al.*, 2013; Egidi *et al.*, 2014).

The aesthetic biodeterioration caused by the development of photosynthetic-based biofilms on the Fishing House glazed tile panel compromises its visual appearance (Fig. 1). Moreover, the extensive microbial colonization by epilithic and chasmoendolithic communities eventually promotes physical damage, such as glaze detachment and cracking. The growth of biofilms on glazed ceramic building materials has been associated with deepening of fractures or detachment of the glaze when chasmoendolithic growth occurs (Palmer and Hirsch, 1991; Giacomucci *et al.*, 2011;

403 Coutinho *et al.*, 2013). In addition to aesthetic and biophysical damage on glazed tile
404 substrates that are hostile for microbial colonization due to their reduced porosity and
405 roughness (Gazulla *et al.*, 2011), the chemical composition of the glaze layer is also
406 worth discussing. Some chemical damage on glazed surfaces has been reported, but
407 little is still known about how microorganisms affect the corrosion of historic glazes
408 (Watanabe *et al.*, 2009; Baricza *et al.*, 2012). The analysed tile panel from the Fishing
409 House was manufactured according to the *majolica* production technique, and therefore
410 it contains a significant amount of lead oxide (Table 5) (Coentro *et al.*, 2012; Coutinho
411 *et al*., 2016). Lead can be lixiviated from the glassy matrix in acid media (Wood and
412 Blachere, 1978). Microbial growth is often accompanied by acid excretion, such as the
413 oxalic acid produced by fungi, which could cause the release of lead from the
414 substratum, making it available for microbial uptake. However, lead is toxic to living
415 organisms and the presence of microbial growth on lead-rich substrates could be
416 unexpected. Yet, the analysed tile panel was severely colonized, and other studies have
417 shown that glazes can be colonized by dense biofilms whenever exposed to certain
418 outdoor environments (Giacomucci *et al.*, 2011; Coutinho *et al.*, 2013). Specific
419 environmental factors, such as cool temperatures, high relative humidity, moderate solar
420 irradiance, shelter from direct rain and surrounding vegetation seem to allow the
421 settlement of microorganisms on ceramic materials (Coutinho *et al.*, 2013, 2015). In
422 addition, some microorganisms can develop in the presence of elements that are
423 considered to be toxic, using survival strategies such as formation of insoluble
424 compounds or EPS (Sayer *et al*., 1995, 1999). The survival of microorganisms in harsh
425 conditions may also involve the formation of mutualistic associations between rock-
426 inhabiting microorganisms (Gorbushina and Broughton, 2009). Gorbushina *et al*. (2005)
427 investigated the interactions between microcolonial fungi and potential photobionts

(algae) under laboratory conditions, proving their ability to develop contact structures through a symbiotic or syntrophic relation, which favoured microbial growth of both partners. A similar form of association might occur among tile-inhabiting microorganisms, corroborating the data reported by Coutinho *et al*. (2013) and the results of the present study, where a close interaction between fungal and algal structures was observed (Fig. 2 and 3c).

Efficacy of the tested biocides

Because microorganisms can damage the substratum on which they are living, in a process known as biodeterioration, it is often necessary to eliminate or control their growth on cultural heritage assets. For this reason, the use of biocides is a common procedure in conservation and restoration interventions. Therefore, a set of commercial biocides frequently used on inorganic building materials, together with a non-conventional TiO_2 nanoparticle biocide were tested on colonized tiles from the Fishing House (Oeiras, Portugal).

The assessment of the efficacy of biocides to be used on cultural heritage assets is based on several variables, such as inactivation efficiency, alteration of biofilm coverage area, durability and effect on the substratum (Ascaso *et al*., 2002; Young *et al*., 2008). Because multiple parameters, such as the biofilm macro- and microscopic morphological features, microbial community composition, physiological characteristics and interaction with the substratum need to be analysed, a combination of different methods was used in the present study to provide a more complete set of data.

PCR-based methods (DGGE and cloning) provided information on the composition of microbial communities and the relative abundances of their components;

however, it is important to note that they are not quantitative techniques. Hence, other methods, such as photographic recording and Chla content by epifluorescence, were included in this study to enhance our understanding on biofilm changes and evolution after biocide treatment. Photographic recording provided information regarding the area, colour and density of the biofilm. Several studies have proven that the colour of photosynthetic-based biofilms is indicative of the physiological state of the microorganisms composing them (Prieto *et al.*, 2002; Sanmartín *et al*., 2010). The results also revealed that the density of the biofilm is indicative of the physiological state of the microorganism. For instance, the Chl*a* fluorescence monitored by epifluorescence microscopy clearly shows that when the density of the biofilm is reduced, the Chl*a* fluorescence decreases. DGGE analysis allowed us to monitor the changes in microbial diversity of the studied biofilms caused by the biocidal treatments. The DGGE profiles of the non-treated biofilm samples (C0 and C4) confirmed the stability of the microbial population during the evaluation of the treatments, ensuring that the major changes observed were caused by the effect of the treatments and not due to other environmental factors (Fig. 7). DGGE is a well-known tool for monitoring microbial populations on cultural heritage assets (e.g. Miller *et al.*, 2009; Qi-Wang *et al.*, 2011; Piñar *et al.*, 2013; Otlewska *et al.*, 2014). However, DNA can persist undamaged after cell death, and consequently DNA-based methods do not provide a differentiation between viable and non-viable cells. In addition, several drawbacks of this molecular fingerprint technique are known, including the fact that in complex microbial communities, each band is not always associated with one single organism, and co-migration can occur (Head *et al.*, 1998; Marzorati *et al.*, 2008). Thus, DNA-sequence analysis is essential to provide insight into the biodiversity of the studied biofilms.

To provide a clear understanding, the data obtained in this study are separately discussed for each biocide, focusing on the current knowledge of the mechanisms of biocidal action, through comparison with other case studies presented in the literature.

Titanium dioxide

TiO_2 is an inert and relatively non-toxic substance that has been tested as an alternative to conventional biocides on mortars and stones exposed outdoors (Fonseca *et al.*, 2010; Pinho and Mosquera, 2011). The biocidal action of TiO_2 nanoparticles has been tested in many laboratory studies (Markowska-Szczupak *et al.*, 2011). TiO_2 nanoparticles (anatase phase based), when irradiated with UV-light, originate electrons in the conduction band and holes in the valence band, enabling catalytic reactions, namely, photocatalysis (Kumar and Devi, 2011). Nanoparticles in contact with water react, producing highly reactive hydroxyl radicals that are able of oxidizing most organic compounds, including cell components (Kiwi and Nadtochenko, 2005; Markowska-Szczupak *et al.*, 2011). The efficiency of TiO_2 is also related to the irradiation source and time of exposure. Generally, several factors influence the antimicrobial activity of biocides, such as the temperature, time of exposure, amount of organic material, growth conditions and growth phase of the microorganisms on which they are applied (Russell, 1999). In fact, the effectiveness of TiO_2 as a biocide has been variable according to the irradiation source used, such as UV-lamps, solar irradiation and solar spectra lamps (Gazulla *et al.*, 2011; Gladis and Schumann, 2011; Portillo *et al.*, 2011). Hence, we decided to test its efficacy under natural environmental conditions.

Neither macroscopic nor microscopic observations of the TiO_2-treated biofilm indicated alterations in the biofilm density, chlorophyll fluorescence or cell structure (cell disruption). The DGGE analysis of the TiO_2-treated biofilm sample after 4 months of application seemed to reinforce the macroscopic and microscopic observations, and

503 no changes to the genetic profile were observed. The antimicrobial activity of TiO_2
504 nanoparticles through photocatalytic activity has been extensively studied and observed,
505 as previously mentioned (Kiwi and Nadtochenko, 2005; Rajagopal *et al.*, 2006). The
506 effect of biocides on complex microbial communities could be inadequate due to the
507 presence of EPS excreted by several microorganisms, which protect them from
508 environmental toxins (Russell, 1999; Stewart, 2002). In addition, the surface properties
509 of the microorganisms could influence the extent of membrane damage during the
510 interactions with the nanoparticles and produce reactive oxygen species (Hessler *et al.*,
511 2012; Planchon *et al.*, 2013). Even if TiO_2 was not lethal to the microorganisms, it was
512 the only biocide that caused significant changes in the biofilm coverage area. The self-
513 cleaning properties of photocatalytic nanoparticles are well known, and the decrease in
514 the adherence of microorganisms has been previously reported on other ceramic
515 materials (Graziani *et al.*, 2013). Pozo *et al*. (2014) associated TiO_2 nanoparticles with
516 the reduction of bacterial biofilms due to the limitation of EPS production. The
517 detachment of the biofilm, visible 6 months after treatment, was likely caused by the
518 self-cleaning effect and EPS reduction induced by the TiO_2 nanoparticles (Fig. 5). This
519 produced an efficient mitigation of the biofilm, which was clearly evident after 2 years
520 (Fig. S3). In the field of cultural heritage, the removal of microorganisms from the
521 substratum is a critical issue. Mechanical processes may result in material loss and
522 surface alteration, which can cause irretrievable losses. Therefore, the results obtained
523 with TiO_2, causing the detachment of the biofilm, are indeed promising for eradicating
524 microbial biofilms on inorganic cultural heritage materials located outdoors.
525
526
527

Biotin

Biotin is a commercial broad-range biocide that has been tested on built cultural heritage (De los Ríos *et al.*, 2012). This biocide is composed of a mixture of n-octyl-isothiazolone and a quaternary ammonium salt (dodecyl-dimethyl ammonium chloride), and is approved for the conservation of cultural heritage monuments by the European Biocide Directive (Cooke, 2002).

After the application of Biotin on the glazed tiles, photographic recording showed the colour and density changes of the biofilm. Microscopic observations aided by epifluorescence corroborated the effect of the Biotin on the microbial communities, revealing an almost complete extinction of Chl*a* fluorescence accompanied by cell disruption (Fig. 6). Quaternary ammonium compounds, such as those present in this biocide, are cationic surfactants that affect the permeability of the membrane, causing its disruption and the denaturation of structural proteins and enzymes (Buffet-Bataillon *et al.*, 2012).

In the ITS and 18S rDNA gene DGGE profiles, some changes in community fingerprints were observed. However, the most intense bands of the non-treated samples were still visible in the biofilm-treated sample (Fig. 7). The amplification of the cyanobacterial 16S rRNA gene did not produced any positive PCR results, probably due to the absence of cyanobacteria after the biocide application. We cannot conclude whether there was only a partial inactivation and some organisms remained viable or if these bands are the result of undamaged DNA from non-viable (or dead) microorganisms. The new bands, which are coincident with the DGGE profile of the Preventol-treated areas, could indicate that re-colonization occurred in this area 6 months after the application. The reappearance of the Chl*a* fluorescence on the biofilm

552 6 months after the Biotin treatment seems to validate this view. This point is further
553 discussed when analysing the Preventol results.
554
555 *Preventol*
556 Preventol is also a commercial quaternary ammonium-based biocide that has been used
557 for many years on cultural heritage assets, including stone monuments and mural
558 paintings (e.g. Ascaso *et al*,. 2002; Nugari *et al.*, 2009). Visual examinations and
559 photographic recordings showed drastic changes of the colour and density of the biofilm
560 on the treated area. However, no variation in the coverage area was observed (Fig. 4).
561 Similar to Biotin, no Chl*a* fluorescence was observed 4 months after the biocide
562 application (Fig. 6). At this time, the DGGE profiles showed that the dominant bands of
563 cyanobacteria, fungi and eukaryotes on the non-treated biofilm were no longer present
564 on the Preventol-treated biofilm (Fig. 7). This indicates a successful antimicrobial
565 activity of the biocide against the major components of the biofilm community. Indeed,
566 several authors have mentioned the antagonistic action of this biocide against biological
567 communities dwelling on stone (Ascaso *et al.*, 2002; De los Ríos *et al.*, 2012; Speranza
568 *et al*., 2012).
569 The construction of DNA libraries confirmed that most of the non-treated
570 biofilm community members were no longer detected in the Preventol-treated biofilm
571 (Tables 2–4). In particular, none of the fungal community members of the non-treated
572 biofilm was detected in the Preventol-treated biofilm, but the microbial communities
573 were more diverse after biocide treatment, as shown in Tables 2-4. *Cryptococcus*
574 species within the Basiodiomycota phylum and *Aureobasidium pullulans* within
575 Ascomycota were the most abundant microorganisms found in the biofilm after the
576 Preventol treatment. Non-lichenized pigmented fungi were shown to colonize rock

surfaces and restored glazed tiles (Gorbushina *et al*., 1993; Diakumaku *et al*. 1995; Pitzurra *et al*., 2001; Gorbushina and Broughton, 2009; Giacomucci *et al*., 2011; Cutler *et al*., 2013). Giacomucci *et al*. (2011) reported *A. pullulans* in a biofilm developing between the ceramic body and acrylic adhesive-treated tile glazes from the façade of the Grande Albergo Ausonia & Hungaria (Venice, Italy). Other filamentous fungi found in the Preventol-treated biofilm, such as *Chalara* sp. and *Phoma* sp., have been described on diverse building materials (Piñar *et al*., 2009; Shirakawa *et al*., 2010).

The prokaryotic biodiversity was also affected by the biocide. A higher biodiversity was retrieved on the treated biofilm 4 months after the application of Preventol. Some of the identified photoautotrophs belong to the *Coccomyxa, Tolypothrix and Anabaena* genera, which are able to grow in diverse climates and substrata, including stone monuments, and some are even human and plant pathogens (Rosa e Silva *et al*., 2008; Macedo *et al*., 2009; Cutler *et al*., 2013). Non-photosynthetic prokaryotes, such as members of the Verrucomicrobia and Proteobacteria phyla, were also identified in the Preventol-treated biofilm after 4 months of the biocide application.

Changes in the microbial profile and the presence of "new colonizers" after biocide treatment are common in microbial ecology studies, in particular when the colonized object under treatment is exposed outdoors. Lixiviation by rain and a consequent decrease in biocide concentration may promote re-colonization of the treated tile surfaces by microorganisms. Moreover, treatments with quaternary ammonium were shown to increase fungal diversity and notably the black fungi community (Martin-Sanchez *et al*., 2012). One of the most notorious cases of re-colonization by fungi was observed in the Lascaux Cave after several treatments with quaternary ammonium derivatives (Bastian *et al*., 2010; Martin-Sanchez *et al*., 2012).

This fungal outbreak was explained by the ability of microcolonial fungi for growing using quaternary ammonium degradation products.

A re-colonization by secondary microorganisms using cell debris and biocide residues as a nutrient source could has occurred, as corroborated by epifluorescence and PCR-based methods. In this case, an early development of photosynthetic microorganisms was detected by DNA-based analysis, and later by epifluorescence (Fig. 6).

Albilex

Albilex is a commercial biocide that has been used successfully by the National Tile Museum in Lisbon to inactivate the biological colonization of glazed wall tiles. Similar to the other tested commercial biocides (quaternary ammonium-based compounds), alkyl-benzyl-dimethyl-ammonium chloride is the main active compound of Albilex. In addition, it contains chromium dihydroxide ($H_2Cr_2O_7$) as an active principle. Kumar *et al*. (2014) reported that this toxic metal ion can act as a photosynthesis inhibitor, but our data did not provide any result corroborating this effect.

On the Albilex-treated biofilm area, neither colour nor density changes were observed, not even chlorophyll fluorescence alterations. In fact, the effects of this biocide on the community profile were not significant, as observed by DGGE (Fig. 7). The visual inspection of the Albilex-treated area after 2 years showed that this biocide treatment was indeed ineffective.

The procedure for the application of the Albilex on biodeteriorated tiles usually involves submerging the tile in the biocide solution. Hence, repeated applications or an application with a poultice to ensure longer action might be necessary to improve its efficacy.

626 Table 6 summarizes the major results obtained in this study, illustrating the
627 effects of each tested biocide and allowing the comparison of the different variables
628 evaluated, namely the visibility of the underlying tile, removal of biofilm from the tile
629 surface, the long-term effects, the viability of the microorganisms, the diversity of the
630 microbial community and the interaction of the biocide treatments with the substrate.
631
632 ***Tile changes after biocide application***
633 For all tested treatments, no perceivable alteration of the glazed surface is associated
634 with them at present. The morphological characterization of the glaze by SEM analysis
635 after exposure to the tested biocide solutions is a common procedure to evaluate the
636 chemical resistance of glazes (Fröberg *et al*., 2007, 2009). The corrosion and fractures
637 detected on the glazed surface were visible on both treated and untreated tiles.
638 Therefore, the applied treatments seemed to not affect the glazed wall tiles from the
639 Fishing House (Fig. 10 and Table 6).
640
641 **Conclusions**
642 The brownish biofilm growing over the glazed wall tiles from the Fishing House was
643 mainly composed of a complex community of fungi, microalgae and cyanobacteria,
644 represented by *Neodevriesiaceae* sp., *Phycopeltis* sp. and *Oocystis solitaria*,
645 respectively. Although no lichens or lichenization process were identified in the biofilm
646 samples, a close interaction between the melanised fungi and algae was observed. When
647 the efficacy of four biocides was analysed on photosynthetic-based biofilms attached to
648 glazed wall tiles, Preventol was the most effective biocide followed by Biotin, as
649 determined by epifluorescence after 4 months of biocide treatment. However, according
650 to DNA-based analyses and cell viability determined by epifluorescence after 6 months,

a re-colonization by “new” colonizers occurred on the glazed tiles treated with Preventol and Biotin. TiO_2 nanoparticles were not as effective as Preventol as far as cell viability is concerned, but they promoted the detachment of the biofilm from the tile surface, unveiling the tile motifs and high brightness of the glaze 6 months and even 2 years after biocide application. This detachment of biofilm and self-cleaning effect of the TiO_2 nanoparticles-based biocide reveal its potential long-term efficacy. Albilex was ineffective under the tested conditions. These results are illustrative of the importance of assessing biodeterioration and biocide mid- and long-term effects on colonized substrates. Further investigations should be performed regarding the combined use of quaternary ammonium-based biocides, followed by the application of TiO_2 nanoparticles to promote both cell inactivation and detachment of biofilms from inorganic building materials.

The multi-proxy approach used for monitoring the efficacy of the tested biocides seemed to be suitable to provide information on their lethal efficacy against the photosynthetic-based community. However, for the non-photosynthetic community, this approach was sometimes inconclusive. Therefore, complementary methods that can provide information regarding the viability of non-culturable microorganisms should be added to the monitoring of biocides, such as RNA-based analysis.

Experimental procedures

Site description and sampling

This study was focused on the glazed ceramic wall tiles from the stair handrails that give access to the artificial pound in the garden of the Fishing House (38º 41´52´´N; 9º 18´40´´W), located in Oeiras, Portugal. Oeiras has a temperate maritime climate

676 characterized by mildly cold temperatures (average annual temperature 16 ºC) and high
677 relative humidity levels (76%). Tiles facing north are covered with a brownish biofilm
678 (Fig. 1), detracting from the aesthetic impression. Besides the aesthetic damage, fissures
679 and flaking were also observed on the colonized areas of the glazed tiles (Fig. S1).
680 Sampling surveys were conducted between August 2012 and March 2013. The
681 biofilm samples were carefully scraped using a sterile scalpel and placed into sterile
682 tubes for molecular biology analysis and culture techniques. In addition, small glaze
683 samples were collected from previously detached areas with a scalpel for chemical
684 characterization, stereomicroscopy and SEM-EDS. Samples for DNA-based analyses
685 were taken before and 4 months after the biocide application.
686
687 ***Chemical and mineralogical characterization of the glaze***
688 To determine the glaze chemical composition, micro-particle induced X-ray emission
689 (μ-PIXE) analysis was performed using an Oxford Microbeams OM150 scanning
690 microprobe. The glaze samples were irradiated in vacuum and the produced X-rays
691 were collected by a 8 μm thick Be windowed Si(Li) detector with a crystal active area
692 of 80 mm^2 and 145 eV resolution. The system configuration allowed efficient detection
693 of low energy X-rays. Operation and basic data manipulation, including elemental
694 distribution mapping, was achieved through the OMDAQ software code, and
695 quantitative analysis was done using the GUPIX software. The quantification was
696 performed on a representative area (500 × 500 μm) of the glaze cross-section.
697 Micro-Raman (μ-Raman) analysis was performed on the glaze surface and
698 cross-sections, allowing mineralogical characterization and the identification of the
699 glaze crystalline compounds. A Labram 300 Jobin Yvon spectrometer, equipped with a
700 He-Ne laser of 17 mW power operating at 632.8 nm was used, as well as a solid state

external laser of 50 mW power operating at 514.5 nm. Spectra were recorded as extended scans and the laser beam was focused either with a 50x or a 100x Olympus objective lenses or with a 50x Olympus ultra-long working distance (ULWD) objective for depth probing. The laser power at the surface of the samples was varied with the aid of a set of neutral density filters (optical densities 0.3, 0.6, 1 and 2).

Morphological characterization of the glaze and biofilms

Stereo- and light microscopy

Small tile fragments from detachment areas were collected and observed by stereomicroscopy using a Leica MZ 16 stereoscopic microscope (Leica, Wetzlar, Germany) equipped with a digital camera. Fresh biofilm samples were directly examined under a Leitz Diaplan microscope with differential interference contrast (DIC) optics. Images were recorded with a DeltaPix Camera (InfinityX).

SEM –EDS

SEM analyses were performed on three distinct samples: (i) polished-cross sections of the glaze for characterizing the tile substrate; (ii) glaze fragments with biofilm for evaluating biofilm-substrate interactions, and (iii) glaze fragments treated with biocides. In this case, the analysis was performed after cleaning the surface with a cotton swab soaked in a water:ethanol (1:1) solution for removing the biofilm. Variable pressure (VP) mode was used for observing the glazed sections and glaze surfaces after the treatments, avoiding sputter coating. Observations under VP-SEM with an energy dispersive X-ray spectrometer (EDS) were performed using a HITACHI 3700N scanning electron microscope interfaced with a Quantax EDS microanalysis system. This system was equipped with a Bruker AXS XFlash® Silicon Drift Detector (129 eV

Spectral Resolution at FWHM - Mn Ka). The operating conditions for EDS analysis were: 20 kV accelerating voltage, 10 mm working distance and 120 mA emission current. For assessing biofilm-substrate interactions, glaze samples were sputter coated with gold and observed under high vacuum, using secondary electron mode and an accelerating voltage of 10 kV.

Isolation and identification of fungi

Biofilm samples for culture procedures were suspended in sterile water and inoculated onto PDA culture medium (Scharlab, Barcelona, Spain). Individual colonies were transferred to fresh PDA plates. Eight axenic cultures, named CP1 to CP8, were characterized in terms of colony development, morphology, and identified by DNA-based analysis. Genomic DNA was isolated from fungi by the following procedure: Fungal mycelium was collected from axenic cultures and placed into 1.5 mL Eppendorf tube containing 500 μL of TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA; pH 8) and glass beads. The mixture was shaken in a cell disrupter (Fast Prep-24, Solon, OH, USA) at full speed for 2 min. The DNA was purified by phenol/chloroform extraction and ethanol precipitation.

The internal transcribed spacer (ITS) regions, including ITS1, 5.8S rRNA gene and ITS2, were amplified using the universal primer pair ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.*, 1990). PCR reactions were performed in a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using the following thermocycling program: 2 min denaturing step at 95ºC, followed by 35 cycles of denaturing (95ºC for 30s), annealing (55ºC for 30s) and elongation (72 ºC for 1 min), with an additional extension step at 72ºC for 10 min at the end. Subsequently, PCR products were separated on 1% (w/v)

agarose gels, stained with SYBR Green I (Roche Diagnostics, Mannheim, Germany) and visualized under UV light. Positive PCR products were sequenced by Macrogen Europe (Amsterdam, The Netherlands). Homology search of the sequences was performed using the BLASTn algorithm (Altschul *et al*., 1990) of the National Center for Biotechnology Information (NCBI).

In situ ***application of biocidal treatments on glazed wall tiles***

Four different treatments were tested on the biofilms growing over the glazed wall tiles. A photocatalyst compound and three commercial biocides were applied on the colonized glazed tiles: i) Titanium dioxide P25 nanocrystalline anatase (TiO_2) with a specific surface area of 50 m^2 g^{-1} and particle size of approximately 20 nm (Degussa, Frankfurt, Germany); ii) Preventol® RI 80 (Preventol), composed of alkyl-benzyl-dimethyl-ammonium chloride and isopropyl alcohol (Lanxess, Leverkussen, Germany); iii) Biotin®T (Biotin), which contains alkyl-benzyl-dimethyl-ammonium chloride, octyl-isothiazolone and 3-iodo-2propynyl-butylcarbamate (C.T.S., Spain), and iv) Albilex Biostat® (Albilex), containing alkyl-benzyl-dimethyl-ammonium chloride and dihydroxide chromium ($H_2Cr_2O_7$) (2:1) (Albishausen, Usingen, Germany).

The TiO_2 treatment was prepared in an aqueous suspension 1% (w/v), which was previously tested on an outdoor cultural heritage asset *(*Fonseca *et al*., 2010). All commercial biocides were applied using the concentrations specified in the manufactures' instructions: 2% (v/v) for Biotin, 2% (v/v) for Preventol and 1% (v/v) for Albilex. Each treatment was applied directly to 3 tiles with a paint-brush until soaking. This experiment was performed in summer (August 2012) to avoid eventual rainy conditions.

776 ***Monitoring of biocide efficacy***

777 *Macroscopic observations*

778 Macroscopic observations were performed by visual inspection and photographic
779 recording using an Olympus C-5060 digital camera (Olympus, Tokyo, Japan), between
780 August 2012 and March 2013, and after two years of the biocide application.

781

782 *Epifluorescence microscopy*

783 The efficacy of the treatments was assessed 4 and 6 months after the experiment by
784 epifluorescence microscopy. Chlorophyll *a* (Chl*a*) autofluorescence was qualitatively
785 analysed when excited with blue light; if the molecule is degraded, red light emission
786 cannot be observed. Biofilm samples from treated and untreated areas were collected
787 with a sterile scalpel and transferred to sterile Eppendorf tubes. The samples were
788 examined with a Zeiss Axioplan 2 light microscope, equipped with a Nikon DXM
789 1200F camera. Images were captured using a 40x Neofluar objective with a B-1A
790 fluorescence filter cube (BP 450-480 nm, FT 510nm, LP 515 nm).

791

792 *DNA-based analyses*

793 The biofilm samples were collected for the identification of cyanobacteria, chlorophyta
794 and fungi before (C0) and 4 months after the application of the treatments: T4 (TiO_2),
795 B4 (Biotin), P4 (Preventol) and A4 (Albilex). For comparison purposes, an untreated
796 biofilm was also collected 4 months after the biocide application (C4). Total DNA was
797 extracted using the FastDNA®SPIN for Soil Kit (MP Biomedicals, France) in
798 conjunction with the FastPrep®instrument, following the manufacturer's protocol.
799 Standard DNA barcodes were used for identification (Hajibabaei *et al*., 2007; Schoch *et*
800 *al*., 2012; Gismondi *et al*., 2013; Lebonah *et al*., 2014). PCR amplifications of

cyanobacterial 16S rRNA gene fragments were performed using the primer pair Cya106F (5'- CGGACGGGTGAGTAACGCGTG A-3') and Cya781R (5'-GACTACTGGGGTATCTAATCCCWTT-3') as described by Nübel *et al.* (1997). The eukaryotic 18S rRNA gene was amplified using the primer pair EukA (5'-AACCTGGTTGATCCTGCCACT-3') and EukB (5'-TGATCC TTCTGCAGGACT-3') (Díez *et al*., 2001). Fungal ITS regions were amplified using the primers previously described in section 2.5. The PCR amplification protocol consisted of a denaturing step of 2 min at 95˚C, followed by 35 cycles of denaturing at 95˚C for 15 s, annealing of oligonucleotides at 60˚C for 12 s (55˚C for Eukarya-specific primers, 50˚C for ITS regions), and elongation at 72˚C for 2 min, following a terminal step of 10 min at 72˚C, and ending at 4˚C. PCR products were separated on 1% (w/v) agarose gels.

Fingerprint analysis by denaturing gradient gel electrophoresis. The PCR products of the biofilm samples C0, C4, T4, B4, P4 and A4 were analysed by DGGE. Nested PCRs were performed using the amplification products obtained with the primer primers Cya106F-Cya781R, ITS1-ITS4 and EukA-EukB as DNA template. For the cyanobacterial 16S rRNA gene fragments, the primers 341F-GC (5'-CCTACGGGAGGCAGCAG-GC clamp-3') and 518R (5'ATTACCGCGGCTGCTGG-3') were used, as described by (Muyzer *et al*., 1993). For the eukaryotic 18S rRNA gene fragments, nested amplification was performed using the primer pair Euk1A (5'-CTGGTTGATCCTGCCAG-3') and Euk516r-GC (5'-ACCAGACTTGCCCTCC-GC clamp-3'), according to Díez *et al*. (2001). For the ITS1 region, the primers ITS1-GC (5'-TCCGTAGGTGAACCTGCGG-GC clamp 3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') were used (Doaré-Lebrun *et al*., 2006). The amplified fragments were run in 6% polyacrylamide gels at 200V for 3.5h with denaturing gradients ranging from 30 to 50 % for cyanobacteria, and 20 to 45 % for

826 fungi. The eukaryotic 18S rRNA gene fragments were run in a 6% polyacrylamide gel
827 with denaturing gradient ranging from 20 to 50 %, at 100V for 16h. All runs were
828 performed in a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA;
829 USA), at 60ºC, in 0.5X TAE buffer. The 100% denaturing conditions correspond to 7M
830 urea and 40% formamide. Gels were stained with ethidium bromide, and images were
831 acquired using a Bio-Rad UV transilluminator (Bio-Rad, Hercules, CA, USA).
832 *Construction of 16S rDNA clone libraries and sequencing analysis*. Based on DGGE
833 fingerprint analysis, the non-treated biofilm (C0) and the biofilm collected 4 months
834 after the Preventol application (P4) were selected for DNA library construction and
835 sequence analysis. PCR products were purified using the JetQuick PCR Purification
836 Spin Kit (Genomed, Löhne, Germany) and cloned with the TOPO TA Cloning Kit
837 (Invitrogen, Carlsbad, CA). Representative clones were sequenced by Macrogen Europe
838 (Amsterdam, The Netherlands) to further determine their phylogenetic affiliations.
839 DNA sequences were edited with the Bioedit v7.0.4 software (Technelysium, Tewantin,
840 Australia) and aligned using MUSCLE (Edgar, 2004). Aligned sequences were
841 clustered into operational taxonomic units (OTUs) using the program DOTUR (Schloss
842 and Handelsman, 2005), based on a 97% sequence identity cut-off. Homology search of
843 the sequences was performed using the BLASTn algorithm (Altschul *et al*., 1990) of the
844 NCBI database. Sequences resulting from this work were deposited in GenBank with
845 accession numbers LN713987 to LN714021.
846
847 *Phylogenetic analysis of fungal communities*
848 Phylogenetic relationships between the fungal strains and representative clones
849 retrieved from the non-treated biofilm and from the Preventol-treated sample were
850 estimated. The Preventol-treated biofilm was selected for DNA sequencing analysis

based on the DGGE patterns. The Neighbor-Joining analyses were conducted using MEGA 5.2.2. Gaps were treated as missing data; the Kimura 2 parameter substitution model was used and bootstrap values were generated from 1000 replicates. The resulting topology was compared with results from other tree building algorithms, including Maximum-Likelihood and Minimum-Evolution methods.

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Table legends

Table 1. Phylogenetic affiliations of eight fungal strains (isolates CP1 to CP8) isolated from the Fishing House glazed tiles.

Table 2. Phylogenetic affiliations of the OTUs obtained from the 16S rDNA cyanobacterial sequences (using the cyanobacterial specific primers Cya106F-Cya781R) of the non-treated biofilm sample (C0) and Preventol-treated biofilm sample (P4) collected from the Fishing House glazed tiles.

Table 3. Phylogenetic affiliations of the OTUs obtained from the ITS sequences (using the primer pair ITS1-ITS4) of the non-treated biofilm sample (C0) and Preventol-treated biofilm sample (P4) collected from the Fishing House glazed tiles.

Table 4. Phylogenetic affiliations of the OTUs obtained from 18S rDNA sequences (using Eukarya-specific primers EukA – EukB) of the non-treated biofilm sample (C0) and Preventol-treated biofilm sample (P4) collected from the Fishing House glazed tiles.

Table 5. Average chemical composition and standard deviation (SD) of the white glaze (n = 4) obtained by micro-particle induced X-ray emission.

Table 6. Summary of the major results obtained, including the visibility of the underlying tile, removal of biofilm from the tile surface, the long-term effects of the tested biocides, the viability of the microorganisms, the diversity of the microbial community and the interaction of the biocide treatments with the substrate.

Figure legends

Fig. 1. Damage and pathologies found on the Fishing House glazed wall tiles. (a) North-facing handrail with glazed tiles covered with brown biofilm; (b) detail of the aesthetic damage caused by biological colonization; and (c) yellow coloured stain after biofilm scraping.

Fig. 2. Representative micrographs of three independent observations of the biofilm samples observed under light microscope: (a) *Phycopeltis arundinacea*; (b) *Phycopeltis arundinacea* cells surrounded by brown hyphae of a dematiaceous fungus.

Fig. 3. Representative SEM images of the biofilm over the glazed tile: (a) algae and fungal hyphae over the glaze; (b) algal thalli with the characteristic margins (arrow); (c) algae and fungal hyphae; (d) glaze section (G) with algae (A) and glaze fragments attached to EPS (arrows); and (e) section displaying algae (A) and glaze (G).

Fig. 4. Photographic documentation of the visual effect of each biocide on the brownish biofilms from the Fishing House glazed tiles after 1 day, 4 and 6 months.

Fig. 5. Flaking and detachment of the TiO_2–treated biofilm after 6 months.

Fig. 6. Epifluorescence microscope images of biofilm samples collected before (non-treated biofilm) and 4 months after biocidal treatment with: TiO_2, Biotin, Preventol and Albilex. The selected images comprise exemplars of three independent observations for each sample.

Fig. 7. DGGE profiles of the biofilm samples collected from the Fishing House glazed tiles before and 4 months after the biocide application: (a) cyanobacterial 16S rDNA. (b) fungal ITS regions. Numbers 1-3 indicate bands corresponding to the isolated fungal strains *Devriesia modesta* (1), *Neodevriesiaceae* sp. 2 (2) and *Neodevriesiaceae* sp. 1 (3). (c) eukaryotic 18S rDNA. Lanes C0 and C4 correspond to samples of the non-treated biofilms collected on the first day and 4 months after the biocide application, respectively. Lanes T4, B4, P4 and A4 correspond to samples collected 4 months on areas treated with TiO_2, Biotin, Preventol and Albilex, respectively.

Fig. 8. Phylogenetic tree derived from ITS1-5.8S-ITS2 regions of rRNA gene sequences showing the relationships between isolated fungal strains and OTU representative clones retrieved from the biofilm samples (names in bold). The closest related sequences to the isolated fungal strains were also included. All nodes of the tree were recovered using Maximum-Likelihood and Minimum-Evolution treeing algorithms. Bar, 0.05 substitutions per nucleotide position.

Fig. 9. Representative VP-SEM images of the glaze showing: (a) a glaze cross-section with inclusions; (b) false coloured elemental distribution map for K, Fe, Pb and Sn; (c)

EDS element map overlayed on a SEM image showing the distribution of Si, Fe, Pb and Sn; (d) X-ray microanalysis spectrum obtained from a glaze section.

Fig. 10. Representative VP-SEM images of the glaze after removal of the biofilms on a non-treated colonized glaze (Non-treated) and a treated glaze (TiO_2, Biotin, Preventol and Albilex). Surface morphology of the glaze and high magnified details of quartz inclusions can be observed.