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SPECIAL ISSUE

Development of a biocidal treatment regime to inhibit biological growths on cultural heritage: BIODAM

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Abstract Existing chemical treatments to prevent biological damage to monuments often involve considerable amounts of potentially dangerous and even poisonous biocides. The scientific approach described in this paper aims at a drastic reduction in the concentration of biocide applications by a polyphasic approach of biocides combined with cell permeabilisers, polysaccharide and pigment inhibitors and a photodynamic treatment. A variety of potential agents were screened to determine the most effective combination. Promising compounds were tested under laboratory conditions with cultures of rock deteriorating bacteria, algae, cyanobacteria and fungi. A subsequent field trial involved two sandstone types with natural biofilms. These were treated with multiple combinations of chemicals and exposed to three different climatic conditions. Although treatments proved successful in the laboratory, field trials were inconclusive and further testing will be required to determine the most effective treatment regime. While the most effective combination of chemicals and their application methodology is still being optimised, results to date indicate that this is a promising and effective treatment for the control of a wide variety of potentially damaging organisms colonising stone substrates.

Keywords Biocide · Cultural heritage · Stone · Photodynamic agent · Biodeterioration

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Introduction

The disfiguring of buildings and monuments by biological growths, particularly on recently cleaned buildings, is a cause of serious concern. Biological growths can be aesthetically disfiguring to stone and are a potential cause of decay in the long term, caused by physical and chemical damage to building materials. While green algal growths or lichens are easily recognised as biological, dark or black soiling is often assumed to be non-biological in origin—interpreted simply as a physical process of deposition of soot and other atmospheric pollutants. This fails to recognise the substantial contribution that microbial biofilms make to soiling build-up (Krumbein 2003). Pigments such as chlorophyll, carotenoids and especially the dark coloured melanins produce very strong discolorations on building stone. The latter are often confused with soiling

and fly-ash so that damage caused by biological processes may be confused with that caused by inorganic soiling.

Biofilms are thin and sticky layers of microbes. They include bacteria, cyanobacteria, algae and fungi in varying proportions depending on levels of moisture, light, nutrients and other factors. Films of extracellular polymeric substances (EPS) (mainly polysaccharides) are secreted by these organisms and build up on surfaces to form extremely complex and heterogeneous bioconstructions which are called biofilms. Biofilms form on any kind of interface where there is sufficient moisture. Biofilms are sticky and trap sediment particles acting as chemical traps for dissolved ions (Schaefer et al. 2001). This extracellular matrix has a variety of functions which are not yet fully understood. It has been suggested that EPS functions as an adhesive, reduces desiccation and provides the organism with a favourable microenvironment (Nicholson 1996, Nicole et al. 1994).

Biofilms are common components of most soiling layers, contributing both to soiling build-up and to resulting damage to substrates. Biofilms affect soiling accumulation because the sticky secretions readily trap particulates (Fig. 1). Rough, permeable surfaces are most receptive to organisms since these provide a large surface area and retain moisture (Warscheid and Braams 2000). Sandstone and limestone therefore tend to support greater algal biomass than polished granites. Some modern building facades are particularly vulnerable to biological soiling if they do not incorporate details to promote rapid water shedding from exposed stonework (Fig. 1).

Biodeterioration processes are usually a result of complex interactions within the ecosystem of the microbial community and its substrate. Organisms may cause damage by extracting nutrients from the substrate, by active penetration below the surface or by secondary damage, such as expansion and contraction of polysaccharide secretions during wetting and drying. Penetration of organisms below the surface may lead to substantial material loss (Fig. 2).

Where building facades have suffered damage by biological growths, biocides may be used to eradicate the problem. However, in the past decades many of the most effective biocides have been banned because of their environmental and health hazards. There is a greater assertion by those involved in cultural heritage protection that the risks of any surface treatment are assessed and that preventative measures, such as environmental control, are employed where possible (Price 1996). Where intervention with a biocide is appropriate, the treatment should be effective and long lasting in order to be cost effective. Many biocides are only effective for a short period of time (6 months–1 year), making frequent re-application necessary. The treatment must be non-toxic to personnel and the public, and cause no harm to treated substrates, especially where frequent re-applications are likely since cumulative damage may occur over many application cycles. Since it is possible that other chemical treatments have been applied to the stone, the compatibility of the biocide treatment with common conservation treatments, such as stone strengtheners, may also be an important issue.

This paper outlines the polyphasic approach taken in the BIODAM project, in which a combination of treatments, including conventional biocides, has been tested. It illustrates how the effectiveness and safety of individual treatments may be enhanced by attacking the organisms on a number of fronts.

Fig. 1 The dark soiling developed along the upper edge of this stonework is largely biological in origin. Particulate soiling adheres to the sticky secretions of algae and other organisms which have colonised regularly wetted areas of sandstone

Fig. 2 Pitting of this sandstone is caused by subsurface growth of organisms. Decay is confined to the area which is most commonly wetted by rainwater

Aims

BIODAM is a European multidisciplinary research and development project which aims to develop a novel biocide combination treatment to inhibit biofilm damage on cultural heritage. While conventional biocide treatments combine one or more biocidal chemicals to achieve the desired effect, BIODAM aims to eliminate biofilms by combining biocides with other chemical treatments (permeabilisers, pigment and exopolysaccharide inhibitors and photodynamic agents) which increase the vulnerability of organisms, leading to success at minimal biocide concentrations.

In this new approach, microbial processes known to contribute to the survival of the biofilm were reviewed and compounds were identified which could interfere with these systems. Biofilms provide powerful means for the organisms forming and living within them to protect themselves against environmental stresses and many biocidal agents (Fux et al. 2003). Several mechanisms have been proposed to explain this phenomenon of biocide resistance within biofilms, including:

- delayed penetration of the antimicrobial agents into the biofilm extracellular matrix (Fux et al. 2003),
- slowing of the growth rate of organisms within the biofilm (Anderl et al. 2003),
- physiological changes brought about by interaction of organisms within the biofilm community (Donlan 2000; Russell 2003; Jabra-Rizk et al. 2004).

The practical implications of biofilm formation are that alternative control strategies must be devised both for testing the susceptibility of the organisms within the biofilm and treating the established biofilm. Effective treatment strategies will incorporate antimicrobials or other agents that have been demonstrated to penetrate and kill biofilm organisms or treatments that target specific components of the biofilm matrix. A better understanding of the in situ biofilm response to selected treatments requires more study and more sophisticated use of biocidal systems and their application.

Permeabilisers

To be effective, biocides have to be able to enter target cells in sufficient concentration to kill the organism. Chemical permeabilisers can be used to increase the permeability of cell membranes and allow easier, more rapid access of biocides so that while the concentration of applied biocide may be reduced, its concentration within the cell remains high enough to be effective.

The main compartment of the bacterial cell, i.e. cell contents (cytoplasm) surrounded by a cytoplasmic membrane and enclosed in a cell wall, occurs in most microbes, though the number and types of cell membranes varies between organisms. The cell wall is a rigid structure outside the cytoplasmic membrane which provides support and protection from dehydration. The cytoplasmic membrane of cells is the critical permeability barrier separating the inside from the outside. In addition, it is often considered as the major target site for biocides (Denyer and Maillard 2002).

It is generally accepted that the bacterial outer envelope is responsible for the different responses to antimicrobial agents. For example, the outer membrane of Gram-negative bacteria and mycobacteria acts as a permeability barrier and is responsible for the intrinsic resistance of these microorganisms to antimicrobial compounds (Nikaido 2003). In Gram-negative bacteria the barrier function of the outer membrane is mainly due to the presence and features of lipopolysaccharide molecules in the outer membrane, though various multidrug efflux pumps also contribute to the resistance of the cells (Nikaido 2003). Chemicals which increase the permeability of cell membranes can therefore be useful in increasing the vulnerability of cells to biocides.

Although the outer membrane of Gram-negative bacteria protects the cells from many external agents, it is possible to specifically weaken it by various agents that disintegrate its lipopolysaccharide layer. Such agents are collectively termed permeabilisers (Vaara 1992). The classical example is the chelator EDTA, which sequesters divalent cations that contribute to the stability of the outer membrane by providing electrostatic interactions with proteins and lipopolysaccharide (Denyer and Maillard 2002; Vaara 1992). A treatment with EDTA will release a large proportion of lipopolysaccharide from the outer membrane, exposing hydrophobic phospholipids, and creating a hydrophobic pathway for certain substances. This is manifested as increased susceptibility to hydrophobic antibiotics (Vaara 1992; Helander et al. 1997) and increased sensitivity to membrane disintegration by detergents (Helander et al. 1997; Alakomi et al. 2003). In addition to chelators like EDTA, a number of other permeability increasing compounds are known, some of which act quite differently (Vaara 1992).

Pigment inhibitors

Aesthetic disfiguration of stone materials is often related to pigments produced by bacteria, cyanobacteria, fungi and algae. Algae and cyanobacteria produce chlorophylls (green) and carotenoids (yellow or orange), while fungi and actinomycetes produce melanins (black or brown), carotenoids and several other pigmented compounds. While photosynthetic pigments produce mainly aesthetical damage to stone monuments, the presence of a protective

melanin layer in the outer cell wall layer of fungi forms an efficient barrier protecting the fungi from unfavourable environmental influences which may include biocides. Pigment inhibitors interfere with the normal production of protective pigments rendering organisms more vulnerable to environmental stresses.

Exopolysaccharide inhibitors

Microbial biofilms are composed primarily of microbial cells and extra cellular polymeric substances (EPS). EPS are generally composed of carbohydrate, protein and acidic sugar components and may account for 50–90% of the total organic carbon in biofilms. Clearly they are of primary importance to the organisms that produce them in such copious amounts. The vast majority of EPS are polysaccharides. Bacteria, fungi and algae produce different types of exopolysaccharides. Most bacteria and cyanobacteria secrete EPS which consist mainly of water and polysaccharides, although there is a nucleic acid, amino acid and protein component (Platt et al. 1986; Decho 1994). Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of Gram-negative bacteria. In the case of some Gram-positive bacteria, such as the Staphylococci, the chemical composition of EPS may be primarily cationic. Much less is known about fungal biofilms although fungal structures are often surrounded by extracellular polysaccharides (Nicholson 1996; Gorbushina and Krumbein 2000; Gorbushina 2003).

Attachment of microorganisms is the first step in biofilm formation on stone and extracellular cementing substances play a part in the adhesion of cells to substrata (Zobell 1943). EPS also influence physicochemical processes such as diffusion, restricting the penetration of charged molecules such as some biocides (reducing their effectiveness), and enhance protection against dehydration. Chemicals which disrupt the production of EPS can therefore interfere with cell adhesion to substrates, increase the uptake of biocides by organisms and increase their susceptibility to environmental stresses.

Photodynamic agents

Photodynamic agents have recently been developed for use in cancer treatments (photodynamic therapy) (Henderson and Dougherty 1992). The BIODAM project is examining how these compounds can be used to destroy biological growths on stone substrates. When activated by light, photodynamic agents produce free radicals which may kill nearby cells or produce damage to their cell membranes. This leaves the cells more vulnerable to other agents, such as biocides.

Methodology

The use of agents in combination with biocides is a promising approach to the problem of treating biofilms. Knowledge of the mechanisms of action of the agents in such a polyphasic treatment assists in designing optimal mixtures. Polyphasic approaches are likely to be superior to single biocide applications at higher concentrations. A range of interactions between various compound combinations and organisms involved in stone substrate colonisation and deterioration (including fungi, algae, bacteria and cyanobacteria) is being investigated in the BIODAM project. The project is evaluating selected treatments in isolation and in combination to achieve an optimised treatment in which a photodynamic agent, permeabiliser, pigment and polysaccharide inhibitor will be combined with a biocide to produce a treatment which will effectively prevent regrowth of fungi, algae, cyanobacteria and bacteria on stone substrates for a substantial period of time.

Experiments include both in vitro and in vivo testing. Biofilms on mineral surfaces are known to possess significantly different biological properties to suspensions of the same organisms and are frequently refractory to conventional treatment (Donlan 2000; Russell 2003).

For a model biofilm, a number of characteristic organisms were selected and their reactions to the substances in vitro were studied. The main objective of the study was the clarification of the general reaction of an entire subaerial biofilm system to the test treatment. For the experimental model, glass slides and stone blocks (Fig. 3) were inoculated with a mixture of target species with or without addition of inhibitory compounds. Possible synergisms between novel compounds were assessed using a variety of combined formulations on target species. In addition, sandstone samples (Locharbriggs and Obernkirchen) have been exposed at test sites in Scotland, Germany and Spain to acquire a natural biofilm which has also been used for testing the effectiveness of combinations of treatment agents (Fig. 4).

Effects on stone are being evaluated to ensure that no damage is caused to the substrate. The resulting treatment methodology should be of low toxicity to personnel and the general public, effective at minimal biocide concentrations and keep surfaces free of biological growth for a substantial period of time.

Permeabilisers

A number of potential permeabilisers with various mechanisms of action have been investigated in BIODAM. Polyethyleneimine (PEI, mean molecular mass 70 kDa), meso-2,3-dimercaptosuccinic acid (DMSA), nitrilotriacetic

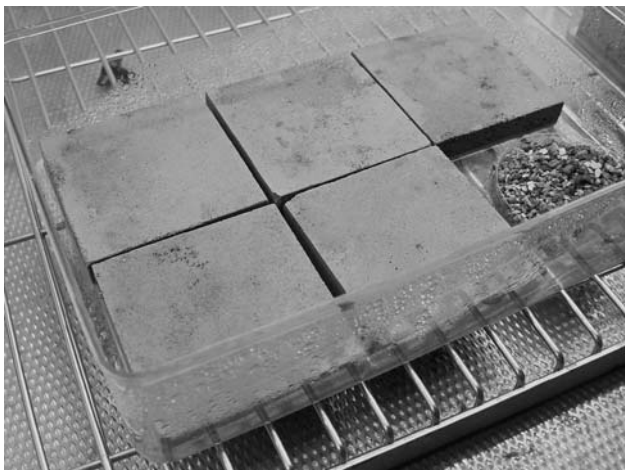

Fig. 3 Organisms, including cyanobacteria as shown here, were cultured on sandstone samples in environmental chambers using vermiculite for humidification. These samples were then used for testing the effectiveness of treatments on organisms on stone substrates

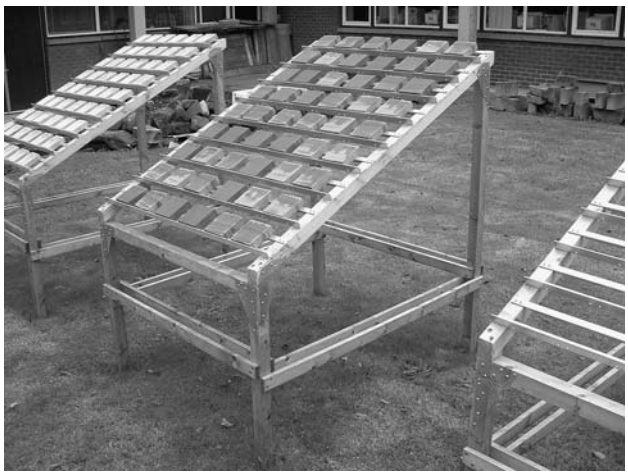

Fig. 4 Sandstone samples exposed at test site in Aberdeen, Scotland to acquire a natural biofilm

acid (NTA) and bis(2-ethylhexyl) sulfosuccinate (AOT) were from Sigma-Aldrich (Steinheim, Germany); and EDTA from Riedel-de-Haen (Seelze, Germany). The activity of each permeabiliser was assessed by measuring fluorescence probe [1-*N*-phenylnaphthylamine (NPN, Sigma-Aldrich)] uptake by bacterial suspensions according to (Helander and Mattila-Sandholm 2000). NPN is utilised in fluorometric studies to measure functional changes of the outer membrane—weakening of the cell membrane allows the penetration of hydrophobic compounds into the cell. Hence, NPN fluoresces strongly in glycerophospholipid environments, but only weakly in aqueous environments. For permeability assays *Pseudomonas aeruginosa* VTT E-96728 cells (obtained from VTT culture collection) were grown in Luria-Bertani broth (LB) as described in Helander and Mattila-Sandholm (2000).

Pseudomonas species are able to degrade chloride compounds and therefore they are not very sensitive to quaternary ammonium compounds (Champanac et al. 2002). In addition, *Pseudomonas* species have been reported to be resistant to many biocides and antimicrobial agents (Russell 2002, 2003). Many Gram-negative bacterial species, e.g. members of *Pseudomonas*, *Stenotrophomonas*, *Sinorhizobium* genera, have been isolated from biodeteriorated stone samples (Saarela et al. 2004). Since members of these genera are potential producers of extracellular polymeric substances (Robertson and Firestone 1992), prevention of their growth or adhesion to stone materials would provide means to diminish biofilm formation on stone surfaces. The origin and history (e.g. previous biocide treatments) of bacterial isolates influences greatly in their susceptibility to antimicrobial compounds.

Although not abundant in stone materials, *P. aeruginosa*, suits well as model bacteria for testing of antimicrobial/potential permeabiliser activity of compounds as these bacteria are generally resistant to many antimicrobial compounds.

Cultivations were carried out at 25°C with shaking (150 rpm). Briefly, cells grown into $A_{630} = 0.5 \pm 0.02$ were deposited by centrifugation at room temperature for 10 min at 1,000*g* and suspended into a half volume of 5 mM HEPES buffer (pH 7.2). Aliquots (100 µl) of this cell suspension were pipetted into fluoroplate wells, which contained NPN (10 µM), and as test substances either EDTA (1.0 and 0.1 mM), PEI (10 µg ml^{-1}), DMSA (1 mM), AOT (1 mM) or HEPES buffer (control) to make up a total volume of 200 µl. If desired, $MgCl_2$ was added to the cell suspension before addition of NPN. Fluorescence was monitored within 3 min from four parallel wells per sample (excitation, 355 nm, half bandwidth 38 ± 3 nm; emission, 402 nm, half bandwidth 50 ± 5 nm). Each assay was performed at least three times.

Pigment inhibitors

A large quantity of work has established the impact of melanin-producing fungi on cultural heritage (Krumbein 2003; Krumbein and Warscheid 1992; Braams 1992; Gorbushina et al. 1993; Urzí et al. 1992). While some of these studies have evaluated the inhibitory capacity of products on microbial stone isolates in the laboratory, pigment inhibitors have not previously been applied to the conservation of cultural heritage. Pigment inhibitors are chemical substances that disrupt enzyme activity and inhibit the formation of pigments in bacteria, cyanobacteria, fungi and algae. Several compounds have been tested in vitro and showed capacity to inhibit the formation of

melanins in fungi by interrupting the melanin synthesis pathway without being toxic to the microorganisms. Tricyclazole, arbutin and carpropamid can disturb the formation of melanins or other dark pigments, showing an inhibitory effect on pigment production in fungi. Cerulenin is a potent inhibitor of carotenoid synthesis in bacteria. Pigment characterisation was performed by photometry after extraction with Soluene 350 and inhibition tests were carried out by using the tricyclazole inhibition assay.

Exopolysaccharide inhibitors

Since EPS is crucial to the formation and survival of the biofilm, EPS synthesis was reviewed in order to identify potential treatments to inhibit EPS production on heritage surfaces. Exopolysaccharide inhibitors include a variety of compounds, including bismuth derivatives, which have inhibitory properties with respect to synthesis of exopolysaccharides by fungi and bacteria. Bismuth antibacterial activity has been enhanced by using certain lipophilic thiol agents, thereby enhancing its inhibitory potency. Although inorganic bismuth is only marginally active for the inhibition of EPS expression (Domenico et al.1996), low levels of bismuth dimercaprol (BisBAL) (Huang and Stewart 1999) have inhibitory activity on *Klebsiella pneumoniae*, which produces the exopolysaccharide alginate. Alginate is one of the most common EPS found in bacteria. The potential of BisBAL to inhibit production of EPS by bacteria and yeast was investigated. EPS production was quantified using the phenol–sulphuric acid method after extraction and solubilisation.

Photodynamic agents

The application of the photodynamic effect towards the destruction of algae and cyanobacteria was investigated. Photodynamic therapy is a method that utilises chemicals which require the application of light for their activity. Photosensitisers under illumination produce radical species and singlet oxygen which have a very short lifetime. The radical species can penetrate the cell wall or cell membrane in a similar action to biocides and attack the cell membrane. Two photosensitisers, methylene blue (MB) (Tuite and Kelly 1993; Misran et al. 1994) and nuclear fast red (NFR) (Reszka et al. 1986) with and without the addition of hydrogen peroxide (H_2O_2), were investigated as having potential in the photo-destruction of algae and cyanobacteria on stone samples. Under UV illumination H_2O_2 undergoes photolysis producing hydroxyl radicals which are powerful oxidisers. The illumination source investigated in this case was a 500 W tungsten halogen white light. The presence of H_2O_2 provides another sink for the singlet oxygen and radical species produced via activation of the photosensitisers. It was anticipated that the singlet oxygen and radical species produced by the dyes would then effect the splitting of hydrogen peroxide producing hydroxyl radical species which would improve the destruction of algae and the breakdown of the dyes. Breakdown of photodynamic agents and activity of algae and cyanobacteria was monitored by measuring their fluorescence.

Experimental results

Permeabilizers

A number of potential permeabilisers with various mechanisms of action have been investigated. Screening studies of chemical permeabilisers [EDTA, polyethyleneimine (PEI), meso-2,3-dimercaptosuccinic acid (DMSA), nitrilotriacetic acid (NTA) and sulfosuccinate (AOT)] was carried out to narrow down the range of potential agents. The performance of these agents was assessed by fluorescence probe [1-*N*-phenylnaphthylamine (NPN)] uptake assay. Figure 5 shows an example of the NPN uptake of a Gram-negative bacterium, *P. aeruginosa*, when treated with a variety of permeabilisers with and without the addition of $MgCl_2$. Increased fluorescence indicates stronger permeabilisation. Figure 5 shows that on treatment with 1 mM EDTA and 2 mM DMSA the outer membrane of the cells was strongly permeabilised. This effect was eliminated or reduced by the addition of 1 mM $MgCl_2$ to the buffer used in the NPN assay. This indicates that membrane disintegrating activity was mainly related to the amount of divalent cations in the membrane structure. Screening studies of chemical permeabilisers revealed

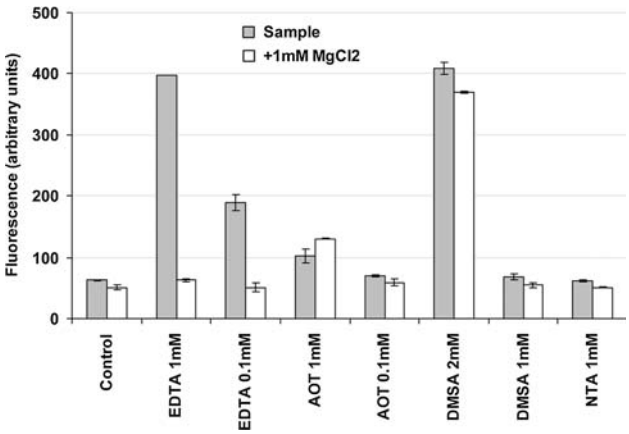

Fig. 5 NPN uptake in suspensions of *Pseudomonas aeruginosa* E-96728. Upon treatment with 1 mM EDTA and 2 mM DMSA the outer membrane of the cells was strongly permeabilised as recorded as an increase in the fluorescence on NPN

EDTA and PEI to be potentially useful permeabilising agents. Alakomi et al. (2006) have reported similar results with bacterial strains (members of *Pseudomonas* and *Stenotrophomonas* genera) isolated from biodeteriorated mineral materials.

Pigment inhibitors

Various isolates from decayed stone were identified and analysed for their inhibitory response to the pigment inhibitor tricyclazole. Fungi were isolated from black crusts on sandstone monuments. The black fungi belonged to *Cladosporium*, *Penicillium* and *Phoma* genera, producing a dark green pigmented mycelium in liquid and solid media. Tests for demonstrating the inhibitory effect of tricyclazole were run on liquid media (Sabouraud Dextrose broth) complemented with 10, 20 or 30 mg/l of tricyclazole (Fig. 6).

Fungi become whitened as the pigment inhibitor prevents normal production of the black pigment melanin. Whitening of fungal hyphae treated with tricyclazole was complete even at 10 mg/l of concentration of tricyclazole. The culture medium turned red due to the accumulation of side products of the normal melanin synthesis. In all the tested strains tricyclazole did not affect fungal growth, showing that the effect of the pigment inhibitor is not a toxic effect—the pigment inhibitor only affects melanin production, not biomass. Organisms can recover their ability to produce melanin within a few weeks, but during that period they are at an increased vulnerability to biocides and environmental stresses.

Exopolysaccharide inhibitors

Results have shown that BisBAL (when coupled to the permeabilisers EDTA and PEI) is able to inhibit EPS production of *Pseudomonas* bacteria, both in vitro and in vivo. BisBAL combined with EDTA and PEI completely inhibited EPS formation in vitro. BisBAL also had a significant inhibitory effect in vivo where EPS production decreased by 45%.

BisBAL was tested for inhibition of extra cellular polymeric substances on a stone isolate Gram-negative bacteria. Inhibition tests showed that the bacteria were inhibited by BisBAL, even at the lowest concentration (5 μM) (Fig. 7). There was a continuous decrease of EPS production from 5 to 20 μM of BisBAL. Higher concentrations did not decrease EPS production further. The effectiveness of BisBAL varied between strains of bacteria. In some, EPS production was unaffected even at concentrations of 50 μM and BisBAL appears not to be effective against Gram-positive bacteria or yeast.

Photodynamic agents

The criteria for selection of photodynamic agents (PDAs) included a high yield of oxidising radicals, no dark toxicity (i.e. the substances are only damaging to organisms under light activation) and the PDA must be easily broken down under visible light. This last factor is important since many PDAs, including the two under investigation [nuclear fast red (NFR) and methylene blue (MB)], are coloured dyes. The rapid decomposition of the PDA after achieving its biocidal effect is therefore important so that no coloured or otherwise harmful residue remains in the substrate. The breakdown of the dye is an essential part of the PDA requirements. If the PDA proves too recalcitrant then this method of algal destruction would result in the introduction of another potential toxin into the environment.

Figure 8 illustrates the effect of the photosensitisers nuclear fast red and methylene blue in the presence and absence of H_2O_2 on the viability of *Synechoccus*

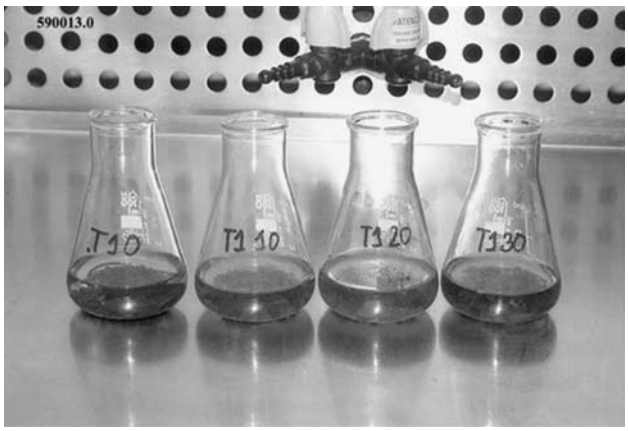

Fig. 6 Whitening of fungal mycelia and darkening of culture media after growth of *Cladosporium* sp on liquid media

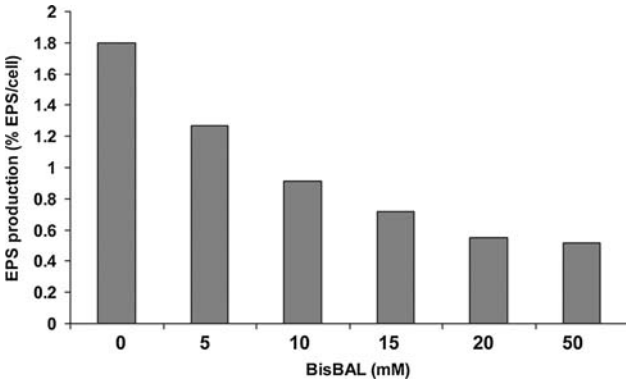

Fig. 7 Effect of BisBAL on EPS production on a Gram-negative bacterial strain isolated from decayed sandstone showing that increasing concentrations of BisBAL result in reduced production of extracellular polymeric substances

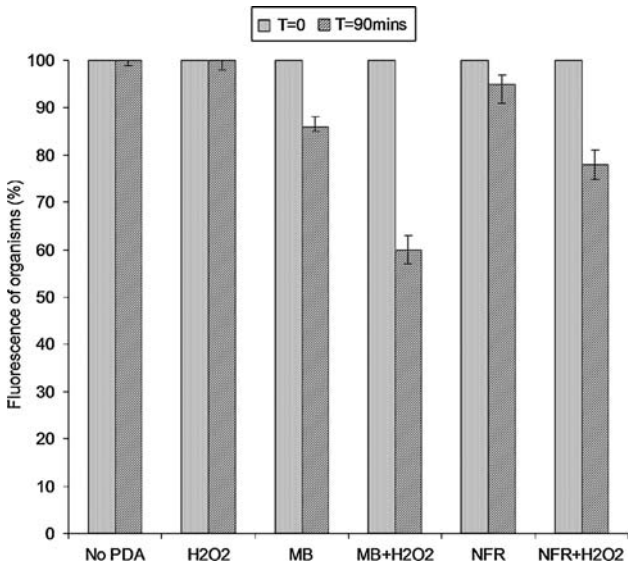

Fig. 8 Effect of methylene blue (MB) and nuclear fast red (NFR) in the presence and absence of H_2O_2 on the viability of *Synechoccus leopoliensis*. *PDA* Photodynamic agent. The effect of illumination on *Synechoccus leopoliensis* alone is also illustrated ('no PDA')

leopoliensis (SL). The fluorescence of the photosensitisers was used to monitor their breakdown (as in Fig. 9) while the fluorescence of the phycocyanin pigment of the cyanobacterium (as in Fig. 8) was used to monitor the viability of *S. leopoliensis*. The irradiated samples were analysed using a luminescence spectrometer (Perkin-Elmer LS 50B). The excitation and emission wavelengths for fluorescence monitoring of cyanobacteria, MB and NFR were 420:685; 667:691 and 545:595 nm, respectively.

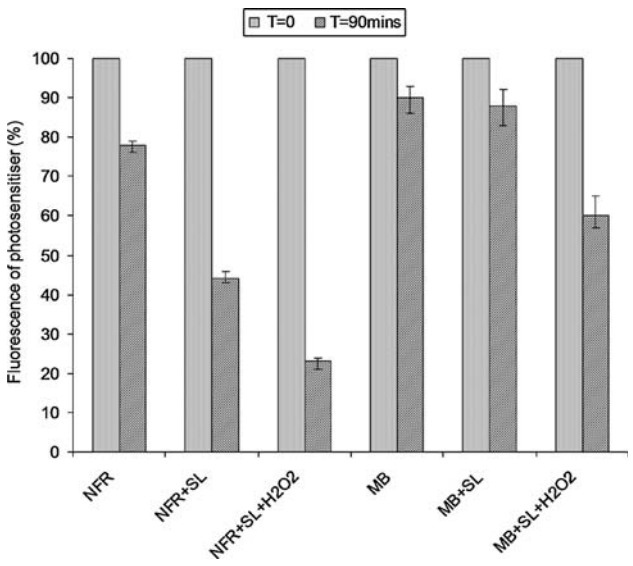

Fig. 9 Plot of fluorescence of photosensitisers methylene blue (MB) and nuclear fast red (NFR) in the presence and absence of *Synechoccus leopoliensis* (SL) and H_2O_2

Illumination alone had no effect on the fluorescence of *S. leopoliensis*, nor did the presence of H_2O_2 cause any change in the fluorescence. When MB was present the fluorescence decreased by 15% over the course of the irradiation time. Combining MB with H_2O_2 resulted in a more significant impact on the viability of *S. leopoliensis*, with the fluorescence decreasing by 40% over the course of the experiment. NFR does not impact on the viability of *S. leopoliensis* as greatly as MB with a decrease in fluorescence of 5% recorded after 90 min irradiation. The presence of H_2O_2 with NFR improved the activity of the photosensitiser with a decrease of 22% in fluorescence of *S. leopoliensis*.

Figure 9 illustrates the breakdown of the photosensitisers over the course of the photo-dynamic destruction of *S. leopoliensis*. The control experiments of illumination of MB and NFR alone illustrated that the light had an effect on both with the fluorescence of NFR (decreasing by 22%) and of MB (decreasing by 10%).The fluorescence of NFR decreased further when *S. leopoliensis* was present suggesting that the radical species produced attack the NFR structure before impacting on *S. leopoliensis*. This would correlate with Fig. 8 where the presence of NFR had little impact on the viability of *S. leopoliensis*. Comparing with the MB results, the fluorescence of MB was little affected by the presence of *S. leopoliensis*. When H_2O_2 was present the fluorescence of NFR decreased to 24% of its original reading, the overall result being that the radical species produced attack the NFR structure prior to attacking the cyanobacterium. The combination of MB with H_2O_2 in the presence of *S. leopoliensis* illustrated a decrease in fluorescence of both MB and *S. leopoliensis* of similar proportions resulting in a good combination for both cyanobacterium destruction and breakdown of the photodynamic agent.

Combination treatments including biocides

Potential synergisms were examined by combining treatments. Synergisms may enhance the performance of the treatments and/or reduce the concentration of active agent required. Combinations of treatments to control biofilm growth were assessed in a polyphasic approach that included conventional biocides at minimal inhibitory concentrations. Tests of treatment efficacy were conducted on cell suspensions, biofilms and on colonised stone surfaces.

The efficacy of cell permeabilisers (EDTA and PEI), the EPS inhibitor, (BisBAL) and a photodynamic agent (NFR) were examined in combination with biocides (benzalkonium chloride (BAC) and Preventol A8^{TM}) to determine their ability to enhance biocide activity. Figures 9 and 10 show results from tests which were carried out in vitro on solid media by agar diffusion tests. Effectiveness was assessed

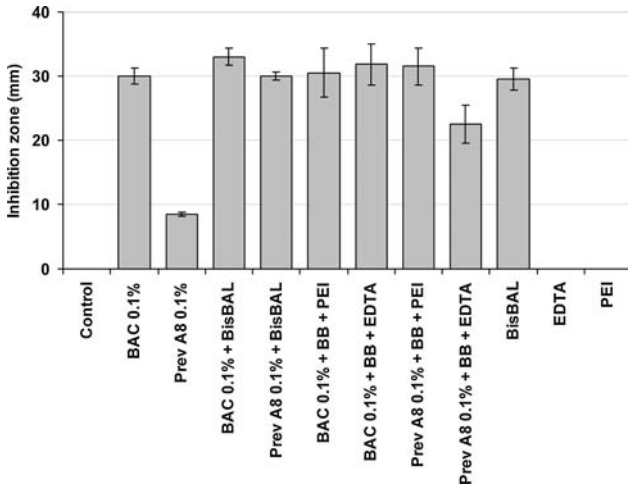

Fig. 10 In vitro tested combination of active substances on the cyanobacterium *Anabaena cylindrica.* Significant inhibitory effects have been achieved by combinations of biocides with an EPS inhibitor and permeabilisers. A. benzalkonium chloride 0.1% B. benzalkonium chloride 0.1% + BisBAL

by measuring the size of the zone of growth inhibition on cultures.

Some organisms were highly vulnerable to all tested treatments, but for others the combination of EPS inhibitor with the biocide was more effective than the biocide alone. In the inhibition tests on *Anabaena cylindrica* the efficacy of Preventol A8^{TM} and benzalkonium chloride treatment was significantly increased by the addition of both permeabilisers and exopolysaccharide inhibitor (Figs. 9, 10). Permeabilisers (PEI and EDTA) were also found to enhance the activity of benzalkonium chloride against the Gram-negative bacterium, *Pseudomonas* sp. In addition, PEI and the pigment inhibitor tricyclazole enhanced activity of the biocide Preventol A8^{TM} against the fungus *Cladosporium cladosporoides* (Fig. 11).

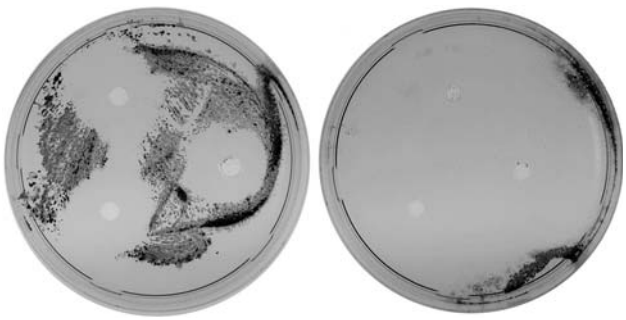

Fig. 11 Agar diffusion tests on a pure culture of a cyanobacterium *Anabaena cylindrica* demonstrate a significant increase of the biocidal effect by the addition of an EPS inhibitor. Plates are initially fully covered by a culture of the organism. Three wells are cut and treatments added to each well. Clear areas indicate zones where cyanobacterial growth has been inhibited

Treatments were field tested singly and in combination on sandstones exposed at field sites and colonised by natural biofilms. The optimised combination treatment chosen included nuclear fast red (photodynamic agent), Preventol A8^{TM} (biocide), polyethyleneimine (permeabiliser), tricyclazole (pigment inhibitor) and BisBAL (extracellular polymeric substances inhibitor). The effectiveness of the treatments was assessed by measuring the fluorescence and colour of the surfaces before and after treatment, as an indicator of photosynthetic growth and by microbiological analysis of selected specimens (generally the best treatment and the control) to examine the effects of the treatments on heterotrophic microorganisms. The performance of treatments was found to be very variable with no consistency in the most effective treatment between test sites or stone types.

Conclusions

Scientific investigations on biodeterioration and decay of European monuments have convincingly shown that hitherto damage functions were often only interrupted or avoided when considerable amounts of potentially dangerous and even poisonous biocides were applied to the surfaces of infected stone materials. The scientific approach described in this paper aims at a drastic reduction of biocide applications by a polyphasic approach, combining biocides, cell permeabilisers, pigment and polysaccharide inhibitors with light activated photodynamic agents.

An efficient treatment must kill damaging organisms and prevent their re-growth for an acceptable length of time while causing no damage to the substrate through biocidal activity or through chemical residues in the stone. Biocides can be hazardous substances, with the potential to pollute the immediate environment of treated monuments. The BIODAM approach is to reduce the health hazard for conservators and the general public through reducing the concentration of biocide required. Treatments developed in the BIODAM project have the potential to provide an environmentally safe method for controlling biological growth on buildings, minimising loss of the built heritage through the action of biofilms and reducing health and safety risks for those involved in its conservation.

The two photodynamic agents investigated here (NFR and MB) have been shown to have the potential to destroy cyanobacteria on stone samples and, since the PDAs are themselves subsequently broken down under visible light, no harmful or coloured residue remains in the substrate. Overall, NFR was found to be a more effective and reliable agent than MB. PEI and EDTA were found to be effective permeabilisers and enhanced the activity of biocides, with

PEI enhancing activity more than EDTA. More detailed results of permeability tests are presented in Alakomi et al. (2006). Tricyclazole was an effective pigment inhibitor in five strains of fungi. The EPS inhibitor BisBAL, especially in combination with a permeabiliser (PEI) was effective at reducing EPS production. Furthermore, the activity of the photodynamic agent (NFR) was enhanced when combined with PEI.

Although treatments proved successful in the laboratory, field trials were inconclusive and further testing will be required to determine the most effective treatment regime. However, combination treatments did display evidence for synergisms that enhanced anti-microbial activity. The techniques of application need to be further developed and tested in order for their role to be fully realised. While the most effective combination of chemicals and their application methodology is still being optimised, results to date indicate that this is a promising and effective treatment for the control of a wide variety of potentially damaging organisms colonising stone substrates.

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