

INHIBITORS OF BIOFILM DAMAGE ON MINERAL MATERIALS (BIODAM)

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Abstract

Scientific approaches to the safeguarding of stone monuments have evolved over the years to reach a high level of sophistication. Analysis and treatment of detrimental biofilms has also made progress. One factor is the awareness not only of biologically initiated chemical but also physical and mechanical damage functions. Another line of evolution of techniques and application proposals is the application of biocidal chemicals. However, in the past 20 years, these have been increasingly banned because of environmental and health hazards produced by these highly toxic substances. In this communication we give an outline of a project, in which chemical, physical, geological and microbiological knowledge is combined in order to find less dangerous ways to eliminate or inhibit detrimental microbial growth on and in rocks and other mineral materials. Special attention is given to the detrimental effects of poikilophilic algal, cyanobacterial and especially fungal films and networks. Biocidal products, including photodynamic treatments, are combined with pigment and exopolysaccharide inhibitors and cell wall permeabilizers to make biofilms vulnerable to chemicals able to kill the associated micro-organisms.

Keywords: biocide, stone, biofilm, pigment, exopolysaccharide, permeabilizer, photodynamic treatment

1. Introduction

Biodeterioration of stone has received considerable attention during the last few years, after recognition of the biological origin of alteration processes suffered by many stone monuments and historical buildings. Observations in the field and experiments in the laboratory (e.g. Gorbushina et al. 1993, Diakumaku et al. 1995; Guillitte and Dreesen 1995, Dornieden et al. 1997, Sterflinger and Krumbein 1997) have demonstrated the significant potential for damage by living organisms.

This project aims to develop novel treatments to eliminate and prevent biofilm colonisation of stone substrates. New approaches to biocontrol include the use of pigment and exopolysaccharide inhibitors and permeabilizers to increase the effectiveness of existing biocide treatments and novel treatments utilising photodynamic agents. Treatments will be tested for their effectiveness in protecting stone surfaces from biofilms and

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evaluated for biodegradability, toxicity and chemical compatibility with substrates. Laboratory based experiments and field trials in Germany, Spain and the UK will be used to evaluate developed treatments under a range of climatic conditions.

2. Background

At the Earth's surface, physical and chemical processes always operate under the direct or indirect control of living organisms. Alteration and breakdown of rocks is also significantly controlled by omnipresent micro-organisms – bacteria, algae and fungi. The development of a growing biofilm on a rock surface considerably modifies its surface structure (Fig. 1a). In such biofilms micro-organisms use structural irregularities of the stone material for their growth. For instance, biofilm growth is stimulated in the presence of locally enhanced moisture availability in cracks and fissures. These favourable environments are preferentially occupied by micro-organisms leading to biofilm growth along crevices (Fig. 1b). Such penetration physically damages stone leading to the development of weathering lesions (Dornieden et al. 1997, Sterflinger & Krumbein 1997).

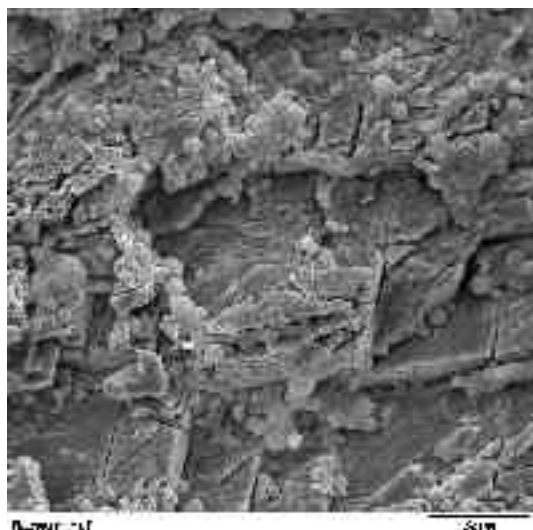


Figure 1a: SEM micrograph of a microbiologically altered rock surface. Abundant growth of algal, bacterial and fungal cells is easily recognisable.

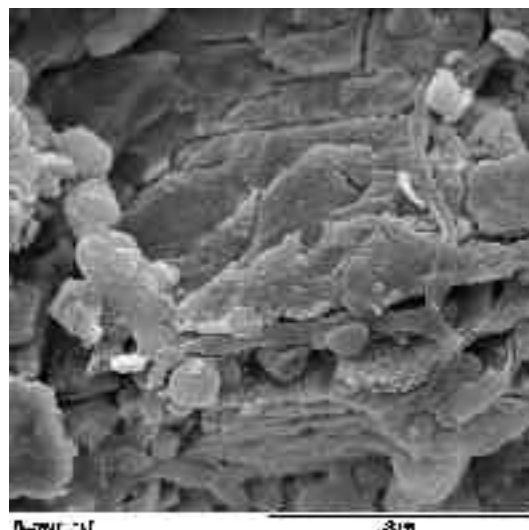


Figure 1b: SEM micrograph of a detail from Fig. 1a, where deeper growth of fungal hyphae (filaments), algal cells (large, round cells) and bacteria (small rods and cocci on the surface of fungal hyphae) in the crevices is observed.

Biologically initiated chemical effects on the surface are frequently connected to the presence of pigments (protective as well as photosynthetic) and extracellular polymeric substances (EPS) that influence the colour (spectral appearance) and structure of the surface. Fig. 2 demonstrates chemically rounded lesions around algal cells, formed under the direct influence of EPS. Initially, biological growth concentrates on the intergranular boundaries, but chemical etching into the rock produces sponge-like structures (reflecting shapes of the colonies) and increases the contact surface between the biofilm, atmosphere and rock material.

The penetration of growing organisms into rock, and the diffusion of their excreted products into the intergranular fissures are likely to lead to enhanced weathering reactions and decreased cohesion between grains to depths which may often exceed 2 cm.

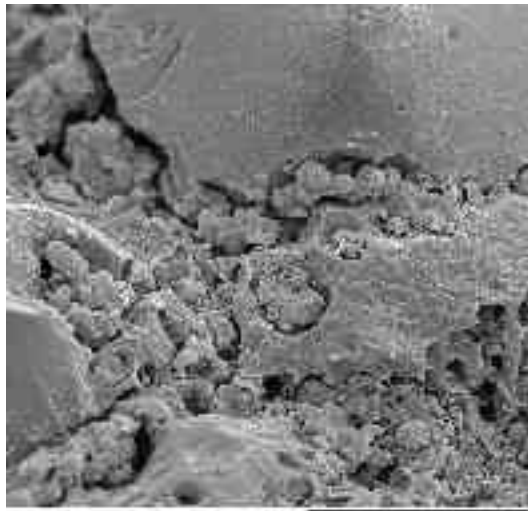


Figure 2. Chemically rounded lesions around algal cells, formed under the direct influence of exopolysaccharides.

2.1 Pigment inhibitors

Microbial biofilm development is frequently associated with biogenic discoloration of the stone material (Gorbushina et al. 1993, Urzi and Krumbein 1994, Koestler et al. 1997). Diverse pigments are produced by rock inhabiting bacteria, fungi and algae. Many fungi isolated from stone have protective dark pigmentation (melanins, carotenes). Photosynthetic micro-organisms like green algae (Chlorophytes) and cyanobacteria frequently settle on stone and abundantly produce photosynthetic pigments necessary for their existence. The development of photosynthetic communities on building materials is often associated with high humidity and water retention, producing a greenish, grey or red biofilm (chlorophyll, carotene). Some groups of bacteria can also produce pigments (e.g. *Pseudomonas*, *Micrococcus* and Actinobacteria). While photosynthetic pigments produce mainly aesthetical damage to the stone monuments, the presence of a protective melanin layer in the outer cell of wall of rock inhabiting fungi (Gorbushina et al., 1993; Gorbushina et al., 2003) forms an efficient barrier protecting microscopic rock inhabiting fungi from the unfavourable environmental influences which may include microbiocidal compounds. Pigment inhibitors are chemicals that inhibit the formation of pigments in bacteria, fungi and algae. *In vitro*, compounds have been shown to have the capacity to inhibit the formation of melanins in fungi (Krumbein and Diakumaku 1995) and certain compounds can produce chlorosis in photosynthetic organisms (isoxazolidones, isoxazoles, pyridazinone).

2.2 Exopolysaccharide inhibitors

A whole spectrum of geomicrobiological interactions between the microorganisms and the rock substrate are mediated by extracellular polymeric substances (EPS) excreted by the growing sub-aerial biofilm (Gorbushina and Krumbein 2000). Attachment of micro-organisms is the first step in biofilm formation on stone, and the adhesion of cells to substrata is due to EPS. Also chemical dissolution of stone may be influenced by acidic polysaccharides present in EPS (Fig.2). Another feature of an EPS layer is the capacity to decrease antimicrobial penetration and diffusion, thus reducing the effectiveness of applied biocides. Bacteria, fungi and algae produce different types of EPS. Chemicals are known which can inhibit the production of EPS, and therefore can be used to inhibit attachment of micro-organisms to stone surfaces. Koulali et al. (1996) demonstrated the inhibitory effect of monensine on the synthesis of EPS by the fungal genera *Botrytis* and *Sclerotium*, and Huang and Stewart (1999) demonstrated the inhibitory effect of bismuth dimercaprol in *Pseudomonas aeruginosa* biofilms.

2.3 Permeabilizers

Permeabilizers are substances that increase the permeability of the cellular membrane of micro-organisms to other substances without being directly microbiocidal. The permeability barrier function of the outer membrane (OM) in Gram-negative bacteria plays a key role in the relative resistance of these bacteria to external compounds that are inhibitory or bacteriocidal towards other types of bacteria, e.g. Gram-positive species. Accordingly, Gram-negative bacteria tolerate high concentrations of hydrophobic antibiotics, detergents, dyes and biocides.

Although the OM of Gram-negative bacteria is an efficient barrier against many external agents, it is possible to specifically weaken the OM by various agents that disintegrate the lipopolysaccharide (LPS) layer. Such agents are collectively termed as permeabilizers. The classical example is the chelator EDTA, which sequesters divalent cations that contribute to the stability of the OM by providing electrostatic interactions with proteins and LPS (Alakomi et al. 2003, Vaara 1999). Although permeabilizing agents may not show a strong bactericidal activity, their use enhances the activity of antimicrobial agents. Chelators are by no means the only permeability increasing substances; in fact a number of other permeabilizers are known, some of which act quite differently. Polyethyleneimine (PEI) is one example of recently recognised permeabilizer (Helander et al. 1997); this substance destabilizes the OM by binding to it without liberation of LPS. Furthermore, certain small terpenoid and phenolic compounds found in herb plants (“essential oil components”) are active upon OM as well (Helander et al. 1998).

2.4 Photodynamic agents

Photodynamic agents are substances that, under activation by a certain wavelength of light, produce oxidizing radicals (Calzavara-Pinton et al. 1996). Light energy activates the chemical agents inducing a series of oxygen dependent chemical reactions that affect cell membranes and eventually cause cell death. Photodynamic agents can be combined with light sources to destroy biodeteriogens in stone materials.

3. Results

3.1 Effects of permeabilizers on Gram-negative bacteria

In addition to measuring LPS release, the sensitization of bacteria to lysozyme and detergents is used to measure alterations in OM function (Helander et al. 1997, Alakomi et al. 2003). Accordingly, a nonpolar hydrophobic probe, 1-N-phenyl-naphthylamine (NPN) is utilized in fluorometric studies to measure functional changes of the OM. NPN fluoresces strongly in glycerophospholipid environments, but only weakly in aqueous environments.

Slime-forming Gram-negative environmental isolates, like *P. aeruginosa*, play a key role in the biofilm formation on surfaces, since they produce extracellular polymeric substances (EPS) that protect micro-organisms from biocides and enhance adhesion of bacteria to surfaces. Fig. 3 shows an example of the NPN uptake of a Gram-negative bacteria, *P. aeruginosa*, when treated with different chelators. A classical example is EDTA, which causes marked liberation of LPS from the OM, resulting in disruption of the permeability barrier function, whereby hydrophobic antibiotics and macromolecules inhibiting or destroying cellular functions are able to reach their targets of action. In NPN uptake assay, cells treated with EDTA showed increased fluorescence when the fluorochrome reacts with glycerophospholipids. Succimer, chemical name meso-2,3-dimercaptosuccinic (DMSA), an active heavy metal chelating agent, was also capable of permeabilizing *P. aeruginosa*. Nitritotriacetic acid (NTA), a complexing agent of the same general type as EDTA, weakly permeabilized *P. aeruginosa* cells.

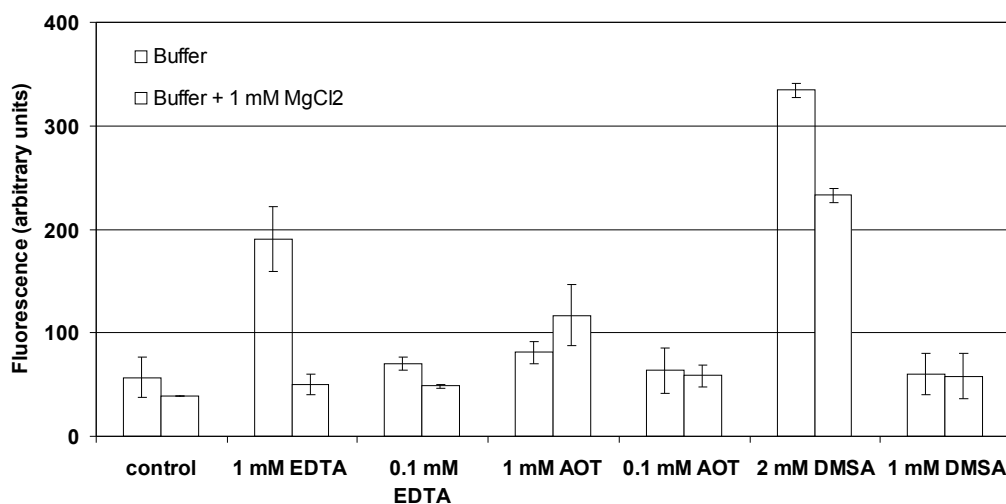


Figure 3: NPN uptake in suspensions of *Pseudomonas aeruginosa* E-97041T. Upon treatment with 1 mM EDTA and 2 mM DMSA the outer membrane of the cells was strongly permeabilized as recorded as an increase in the fluorescence on NPN. Addition of 1 mM MgCl₂ to the buffer used in the NPN assay abolished the permeabilizing activity of EDTA and the NPN uptake of target cells was at the same level as in the control cells.

3.2 EPS inhibitors

Extracellular polysaccharide inhibitors were selected from the literature survey and were tested against a number of bacterial and fungal isolates. Isolation of bacteria was undertaken on sandstone monuments and buildings. Isolates were identified and characterised for EPS production. EPS inhibition tests were carried out by comparing EPS production (photometric method).

A compound called BisBAL (a mixture of bismuth nitrate and dimercaprol) was found to be a potent inhibitor of EPS production on Gram-negative bacteria isolated from decayed sandstone. Its effects have not yet been assessed on Gram-positive bacteria and yeasts.

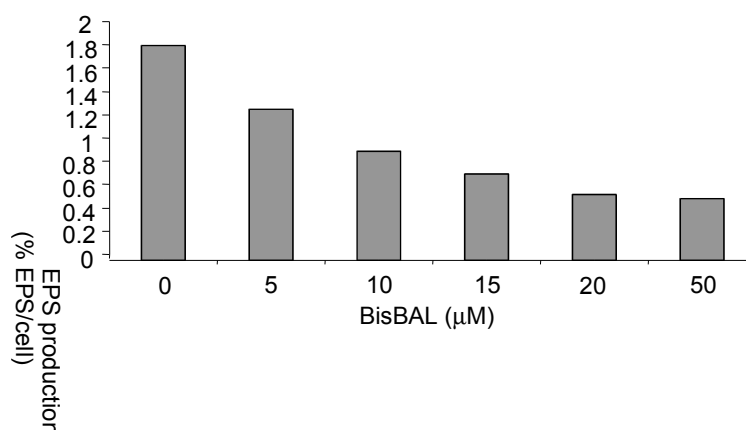


Figure 4: Effect of BisBAL on EPS production on a Gram negative strain isolated from decayed sandstone.

3.3 Photodynamic agents (PDAs)

A selection of photosensitisers (photodynamic agents) was chosen from literature and investigated as potential photodynamic agents in the destruction of algae and cyanobacteria on stone samples. The criteria for selection of photosensitisers are as follows: they must

have an increased absorbance in the red region of visible light, a high quantum yield of triplet formation, they must exhibit no dark toxicity and they must also be easily broken down under visible light. The photosensitisers screened to date include Crystal Violet, Congo Red, Eosin B, Methylene Blue, Nuclear Fast Red, Riboflavin, Rose Bengal (tab.1).

The method employed for monitoring of algae breakdown and dye breakdown is fluorescence. From these results it became apparent that neither Congo Red, Crystal Violet nor Eosin B fluoresce and therefore cannot be used as their breakdown cannot be monitored. 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 to the environment.

Table 1: Excitation and emission data for photodynamic agents

Dye	Excitation (nm)	Emission (nm)
Crystal Violet	-	-
Eosin B	-	-
Methylene Blue	667	691
Nuclear Fast Red	545	595
Congo Red	-	-
Riboflavin	395	485
Rose Bengal	525	559

Nuclear Fast Red (NFR) has been applied to initial experiments of *in vitro* testing of algal samples. The first algal species investigated was *Chlamydomonas nivalis*. This experiment was monitored using a hand held fluorimeter which was designed and constructed in-house for a previous EU project (ONSITE). The fluorimeter was designed to measure low amounts of fluorescence from chlorophyll *a* containing organisms. Its ultra-bright LED excitation source delivers a narrow band excitation wavelength of 430 nm. The emitted light from an excited specimen is collected via lenses and focused onto a photodiode after filtering to remove all but a narrow waveband peaking at 685 nm. The intensity of the emitted light is shown as an output voltage on an attached signal display unit.

To improve the breakdown of the PDAs addition of H₂O₂ to the reaction was carried out. The photolysis of H₂O₂ produces OH radicals which are powerful oxidisers, which will react with the dye molecules. Algae breakdown is affected through singlet oxygen and other radical species produced by the PDAs upon light activation. The addition of H₂O₂ should cause breakdown of the PDAs and, possibly, mineralization of the dyes.

Figure 5 illustrates the effect of hydrogen peroxide on breakdown of *C. nivalis* under illumination with an 8W lamp over a period of 120mins. The presence of H₂O₂ causes an increased reduction in the fluorescence recorded for the algae suggesting that the H₂O₂ is photolysing and the photolysis products are initiating an increased breakdown of algae.

Figure 6 illustrates the effect of NFR on algae breakdown and NFR with H₂O₂ on algae breakdown. The presence of NFR causes an increased breakdown of algae when compared to the results without. After 50mins the presence of NFR causes a decrease of fluorescence of 40%, without added NFR this decrease is only 27%. The presence of H₂O₂ effects a further decrease of 10% of the fluorescence of algae.

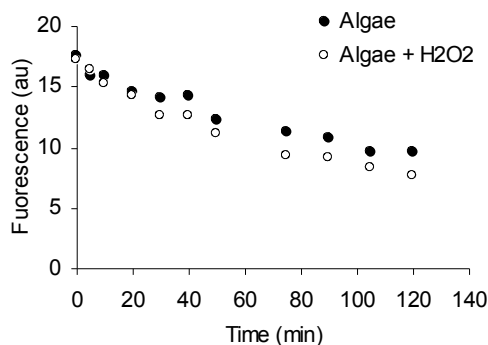


Figure 5: Effect of H₂O₂ on breakdown of *Chlamydomonas nivalis* under illumination of 8W lamp.

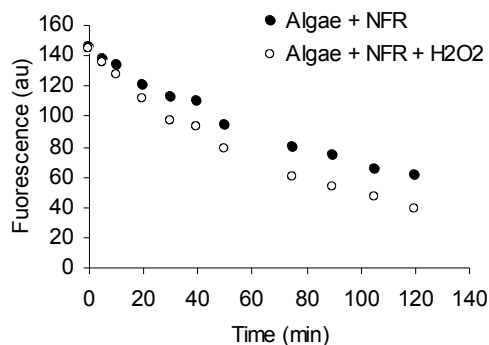


Figure 6: Effect of NFR + H₂O₂ on breakdown of *Chlamydomonas nivalis* under illumination of 8W lamp.

4. Conclusions

Understanding the mechanisms of action of biocides, together with the factors influencing their activity, has become a key issue for a better utilisation of biocidal formulations and control of the emergence of resistant micro-organisms (Maillard 2002). These phenomena are nowadays important in all areas of life where antimicrobial agents are used, whether it is clinical, environmental, surface or process application. In order to be able to enhance the activity of biocides, e.g. with permeabilizers, pigment or EPS inhibitors, the mechanisms and factors influencing their activity has to be understood. New photodynamic treatments and permeabilizers help to reduce the concentrations of otherwise hazardous biocidal treatment agents.

Acknowledgements

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