Control of biofilm growth through photodynamic treatments combined with chemical inhibitors: *in vitro* evaluation methods

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ABSTRACT: The rock/atmosphere interface is inhabited by a complex microbial community including bacteria, algae and fungi. These communities are prominent biodeterioration agents and remarkably influence the status of stone monuments and buildings. Deeper comprehension of natural biodeterioration processes on stone surfaces has brought about a concept of complex microbial communities referred to as "subaerial biofilms". The practical implications of biofilm formation are that control strategies must be devised both for testing the susceptibility of the organisms within the biofilm and treating the established biofilm. Model multi-species biofilms associated with mineral surfaces that are frequently refractory to conventional treatment have been used as test targets. A combination of scanning microscopy with image analysis was applied along with traditional cultivation methods and fluorescent activity stains. Such a polyphasic approach allowed a comprehensive quantitative evaluation of the biofilm status and development. Effective treatment strategies incorporating chemical and physical agents have been demonstrated to prevent biofilm growth *in vitro*. Model biofilm growth on inorganic support was significantly reduced by a combination of PDT and biocides.

Introduction

Micro-organisms play a significant contributing role in the biodeterioration of stone monuments [Warscheid & Braams, 2000; Lamenti et al., 2000]. A considerable number of investigations have started to elucidate the essential role biological agents play in the deterioration of stone [Urzi & Krumbein, 1994; Bock & Sand, 1993]. What is becoming clear is that many factors affect the durability of stone. Physical chemical and biological agents act in co-association, ranging from synergistic to antagonistic effects, to deteriorate stone [Koestler et al., 1994]. The economic impact of materials deterioration is one of the main problems in many developed countries causing losses of 2-4% of the European Union G.D.P., with microbial biodeterioration being responsible for 30% of these losses [Wakefield & Jones, 1998]. Biological deterioration is even more evident in those materials exposed to an outdoor environment, construction materials being the main target of this phenomenon. Stone surfaces have been traditionally treated using physical or chemical methods such as sand blasting or application of chemical biocides. In the past 20 years, chemical biocides have been increasingly banned because of the environmental and health hazards associated with these toxic substances [Warscheid & Braams, 2000, Diakumaku et al., 1995]. Several external pressures including the approval of the European Directive [98/8/EC] concerning placing biocidal products on the market, and the 7th Amendment to Directive [67/548/EEC (Directive 92/32/EEC)] have accelerated the search for more environmentally and toxicologically safe, selective and effective biocides. Photodynamic therapy (PDT) is a method that utilises chemicals that require the application of light for their activity [Henderson & Dougherty, 1992]. PDT has most commonly been used in medical applications where PDT agents are site non-specific drugs, i.e. they do not target a specific enzyme or receptor [Boyle & Dolphin, 1996].

In this work a combined use of PDT agents with low concentrations of traditional biocides was tested on a model biofilm community. Bacteria, algae and fungi involved in stone deterioration were combined into a biofilm representing the diversity of natural communities.

Methods

Model stone deteriorating biofilms

Biofilms for the experiments were grown on a mineral support to simulate the structure of a natural stone- deteriorating biofilm growing on a substrate surface. Such experiment design allowed for devel-

opment of an intensively growing biofilm of active cells belonging to all major groups of a rock inhabiting microbial community. Mixed culture of microbes allowed for testing efficacy of antimicrobial treatments on an *in vitro* biofilm developing at an interface between an inorganic support and a medium.

The mixed microbial culture comprised of *Synechoccus leopoliensis* A591, *Anabaena cylindrical* A490, *Chlorella vulgaris* A60, *Exophiala spinifera* ECa, *Cladosporium* sp. LIIa, *Debaryomyces hansenii* VTT C-04610, *Streptomyces* sp. VTT E-042677 and *Stenotrophomonas* sp. VTT E-022107. Majority of the target microbes were isolated from biodeteriorated surfaces. Starting level of the inoculum was adjusted to 10^3 cfu ml^{-1}. Inoculum was prepared in BG-11 medium supplemented with 0.01 % (v/v) Potato dextrose broth and 0.01 % (v/v) Trypticase Soya Broth.

In vitro tests

Tests were carried out in 24 well microtiter plates (total volume 2 ml) in duplicate. Each well contained a sterile glass coverslip, which served as a mineral support for biofilm development and could be removed after treatment for scanning electron microscopy (SEM) studies. Aliquots of inoculum and tested agents were pipetted directly into the wells. Inoculum was allowed to develop overnight and then tested agents and their combinations were added to the wells.

Antimicrobial treatments

Efficacy of cell permeabilizers EDTA and polyethylenimine (PEI), extracellular polysaccharide formation inhibitor BisBAL [Huang & Stewart, 1999], as well as photodynamic agent nuclear fast red (NFR) alone and in combination with hydrogen peroxide was assessed on their enhancement of the activity of the biocides benzalkolium chloride (BC) and Preventol A8 [Alakomi et al., 2004].

Treatment efficacy evaluation

The viability of the phototrophic microbes was monitored using a hand held fluorimeter which was designed and constructed in-house by RGU for a previous EU project, ONSITE: SMT 4982262. The hand-held fluorimeter was designed to measure low amounts of fluorescence from chlorophyll a containing organisms. The 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 685nm. The intensity of the emitted light is shown as an output voltage on an attached signal display unit.

Viability of microbes was measured by viability staining with LIVE/DEAD® BacLight™ Bacterial Viability Kit (L-7012, Molecular Probes, Inc. AA Leiden, The Netherlands). The staining and fluorescence measurement in microplate scale was performed using the automated fluorometer Fluoroskan Ascent FL (LabSystems, Helsinki, Finland) and black 96-well fluorescence microplates (LabSystems, Helsinki, Finland). Staining solution was prepared according to manufacturer's instructions. The stock solutions dissolved in dimethyl sulfoxide were of the following concentrations: 3.34 mM for SYTO9® and 20 mM for PI. Since PI has a higher affinity for DNA than SYTO9 it is able to displace SYTO9 from the DNA. As a result, viable cells will appear green and dead appear red (damaged cells can sometimes appear orange). For fluorescence measurement samples were taken from microtiter plate wells, harvested by centrifugation and resuspended in 10 mM PBS (pH 7.2) in half volume. Thereafter 100 µl of each microbial cell suspension was pipetted in two parallels into separate wells of a 96-well microplate. Aliquots of 100 µl of the 2x staining solution were added to each well and mixed thoroughly. The plate was then incubated at room temperature in the dark for 15 min and the fluorescence of the bacterial suspensions measured with Fluoroscan Ascent FL fluorometer. Integrated intensities of the green (538 nm) and red (620 nm) emission of suspensions excited at 485 nm were obtained with dual measurement.

Glass coverslips from the bottom of the wells were removed from the wells, air-dried, coated with gold (Balzers Union SCD 030) and examined in Hitachi scanning electron microscope S-3200N. The accelerating voltage was 18-20 kV. Biofilm development was measured on SEM photos of standard magnification by image analysis. The resulting data of biofilm coverage gave an overall assessment of the biofilm development under the influence of combined and single treatments.

Results and discussion

Microscopic and physiological assessment of biofilm development offers a promising method for a better understanding of the *in vitro* biofilm response to selected treatments. The treatments applied were influencing the biofilm activity as well as growth and spreading of the biofilm on the underlying substrate.

This novel experiment design allowed for development of an intensively growing biofilm of active cells which were easy to observe and evaluate in their activity and biofilm formation properties. Mixed culture of microbes allowed for testing efficacy of antimicrobial treatments on an *in vitro* biofilm developing on an inorganic surface. A longer experiment would present a possibility to observe biodeterioration properties of model biofilms.

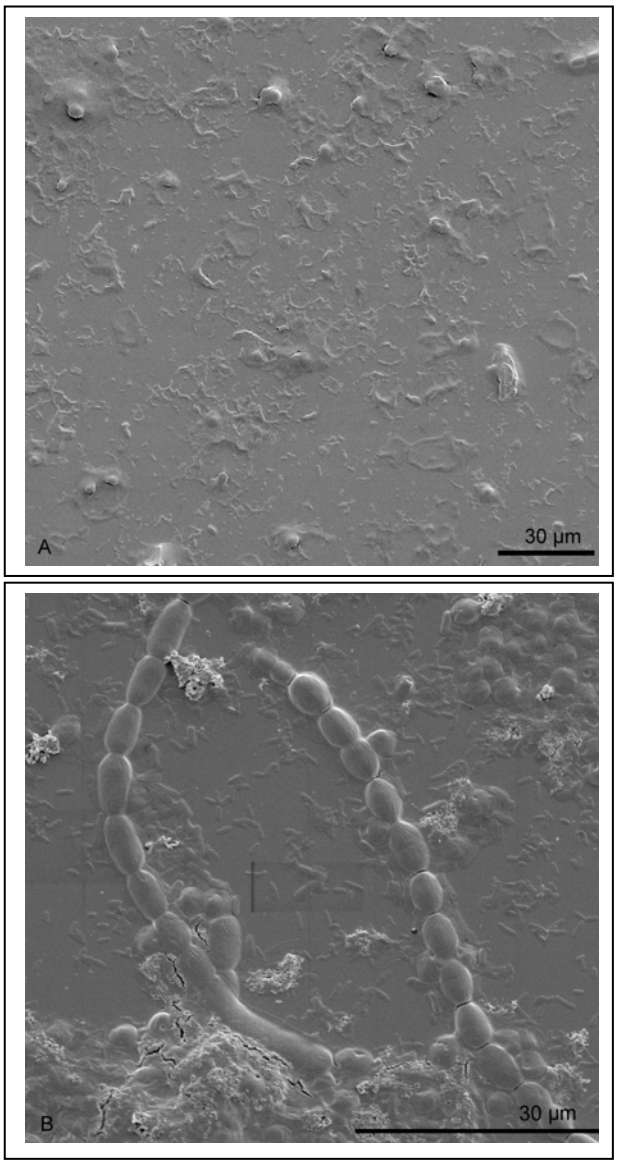

Figure 1: Scanning electron micrograph of a model biofilm on an inorganic support formed under the influence of a biocide/PDT treatment (A). The general structure of the biofilm was significantly deteriorated by the treatment. Number of cells on the surfaces was reduced, significant modifications were observed on the structure of biofilm matrix. B – control inoculum developed in the same time frame without a treatment demonstrates high numbers and diversity of microorganisms embedded in a slimy matrix.

Microscopic studies have convincingly demonstrated that biofilm structure was altered by all treatments (Fig. 1). Number of cells on the surfaces was reduced and significant modifications were observed on the structure of biofilm matrix. A higher magnification allows to observe an undisturbed structure of a complex model biofilm, where organisms maintain a close contact with the underlying substrate as well as with each other.

These results were further supported by measurements of phototrophs' autofluorescence (Fig. 2).

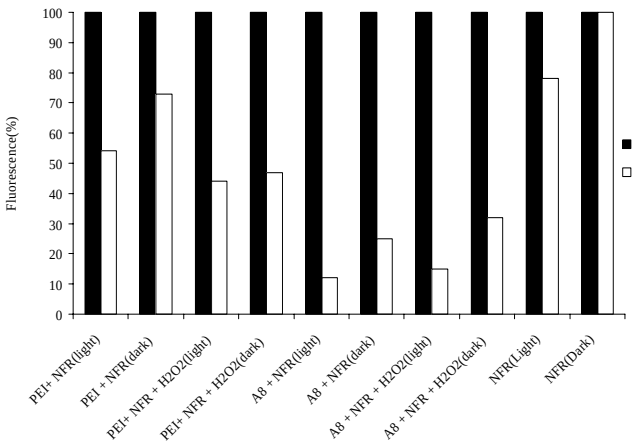

Figure 2: Fluorescence of biofilm after treatment with PDT NFR in combination with a permeabiliser PEI and a biocide Preventol A8.

Figure 2 illustrates the change in fluorescence of the biofilm after illumination from a 300W tungsten halogen bulb when treated with a photosensitiser (NFR) combined with A8 (biocide) and PEI (permeabiliser). The results under both dark and light conditions are illustrated. The photosensitiser in use (NFR) is non-toxic in the dark. It affects a 22% decrease in the fluorescence of the biofilm when applied with light and alone. When it is combined with the permeabiliser there is an affect under both dark and light conditions. A decrease in the fluorescence of 28% was observed under dark conditions when the biofilm was treated with NFR and PEI. Under light conditions an increased effect was recorded, when the fluorescence decreased by 45%. Adding H_2O_2 to the PEI/NFR combination caused even a greater decrease in the fluorescence with very minor difference in efficacy under dark and light conditions.

Combining the photosensitiser with the biocide (A8), caused a further decrease in the autofluorescence of the biofilm under dark and light conditions. The fluorescence decreased by 87% under light activation and by 75% under dark conditions. Adding H_2O_2 to the combined treatment of NFR/A8 reduced the effect observed without its presence. Figure 2 demonstrates that the activity of the photodynamic effect is enhanced when NFR is combined with a permeabiliser or a biocide. These results suggest that combining NFR with a permeabiliser or biocide causes toxicity of the NFR in dark, which does not occur when it is applied alone. The presence of permeabiliser or biocide makes the cell wall more accessible for NFR. Once inside the cell and under activation by illumination, NFR causes damage to the intracellular organelles (chloroplasts or possibly mitochondria), thereby reducing the cell viability and preventing further growth and development.

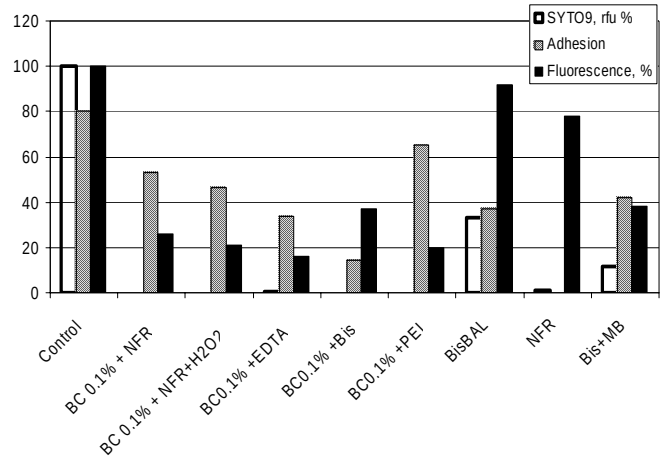

Figure 3. Model biofilm development *in vitro*, as influenced by novel biofouling inhibitors and their combinations.Biofilm growth and activity was assessed by a polyphasic approach including vitality parameters as chlorophyll autofluorescence and Live/Dead stain, as well as biofilm coverage (adhesion).

Figure 3 demonstrates the changes in biofilm growth and development recorded by autofluorescence of phototrophs involved, by percentage of adhered biofilm remaining on the coverglass after removal from the solution and by a vitality nuclear stain SYTO9. Viability results obtained with Live/Dead staining were in agreement with colony forming units results obtained with plate counts for heterotrophic bacteria and fungi (data not shown). All growth and vitality parameters show considerable reduction by treatment combinations. All treatments successfully hamper the biofilm development as compared to control, but their sensitivity are seemingly different, which makes a polyphasic evaluation necessary.

Conclusions

- Model biofilms consisting of deterioration-relevant strains present an effective instrument of assessing a treatment effect *in vitro*.
- Live/Dead viability kit was applicable for estimation of viability of model mixed cultures.
- A polyphasic assessment, combining scanning microscopy with image analysis applied along with traditional cultivation methods and fluorescent activity stains, allows for a broad evaluation of the biofilm status and development. Biofilm growth and viability after various treatments is judged by this approach in a quantitative way using chemical, optical and image analysis techniques.
- Treatment strategies incorporating chemical and physical agents have been demonstrated to prevent biofilm growth *in vitro*. Model biofilm growth on inorganic support was significantly reduced by a combination of PDT and biocides.

Acknowledgements

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