THE APPLICATION OF MODERN TECHNOLOGY IN THE BIODETERIORATION AND PRESERVATION OF BUILT CULTURAL HERITAGE

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INTRODUCTION

This contribution seeks to cover the ways in which microorganisms can be involved not only in the deterioration but also the conservation of stone cultural heritage materials. The topics covered include modern methods for identifying microorganisms and their activities in biofilms; biological treatment of stone ('bioconservation'); and modern methods for treating and preventing colonization and recolonization.

MODERN METHODS FOR IDENTIFYING MICROORGANISMS AND THEIR ACTIVITIES IN BIOFILMS

Identification of the type and activity of microorganisms involved in biodeterioration is necessary in order to understand and control their effects on cultural assets. Recent investigations have focused on the development of qualitative and quantitative molecular tools and latest developments in this area have been reviewed by Sanmartín *et al.* (2016).

Most modern identification techniques involve nucleic acid sequencing. Ettenauer *et al.* (2014) developed a real-time PCR (qPCR) method to detect and quantify fungi present in materials used in the construction of historic buildings. Samples of thermal insulation materials were inoculated with *Cladosporium cladosporioides*, *Aspergillus niger* and *Penicillium chrysogenum*, and parchment samples from the 13th to the 19th century were used as original materials. Villa *et al.* (2015) employed 16S rRNA and sulfur metabolism genes to detect metabolically active microorganisms and provide insight into the diversity of sulfur-oxidizing bacteria in limestone tombstones from two differently polluted sites in urban (more polluted) and rural (less polluted) areas in Massachusetts, USA. The authors used denaturing gradient gel electrophoresis (DGGE) fingerprints of 16S rDNA, 16S rRNA, *soxB*, *aspA* and *dsrA* transcripts to demonstrate that Cyanobacteria was the main metabolically active group at both sites. Higher levels of phototrophic sulfur bacteria, other sulfate-oxidizing bacteria and strains within the sulfate-reducing genus *Desulfovibrio* occurred at the more polluted site.

Next-generation sequencing (NGS) is utilized for the analysis of biofilms on historic and cultural buildings. The ability to use small samples and analyze entire communities without using culture-dependent techniques is extremely attractive and the literature generated from the use of this technology is expanding rapidly. Halophilic bacteria are increasingly found in culturally important buildings. Salt efflorescences are common on stone and other lithic materials, and the use of NGS technology has allowed ready detection of these 'salt-loving' organisms, which frequently belong to the domain Archaea (Piñar *et al.* 2009; Ogawa *et al.* 2017; Otlewska *et al.* 2017). As they are often pigmented, they play an important role in the discoloration of surfaces.

Pigmented bacteria, such as *Rubrobacter* spp., and fungi, such as the Dothideomycetes, are frequent colonizers of stone heritage items (Duan *et al.* 2017; Gaylarde *et al.* 2017a, b). Gaylarde *et al.* (2017a) reported the presence of microorganisms with important survival and deteriogenic properties, such as pigment production, halophily and thermophily, in biofilms on historic churches in Rio de Janeiro, Brazil. These technologies have drawbacks, however (Ogawa *et al.* 2017b), and improvements can certainly be expected in the future. One of the many problems is the lack of discrimination of fungal populations on the stone (Sohlberg *et al.* 2015; Gaylarde *et al.* 2017a).

The activity, rather than the mere identity, of microbial cells in biofilms on the surfaces of historic buildings is a subject of obvious interest. The analysis of potentially deteriogenic activities within the biofilm would greatly increase our capacity to design control measures against biodeterioration. As of yet, methods for the determination of such activities directly within the biofilm are insufficiently developed. However, an initial study was made on buildings of the Auschwitz II-Birkenau concentration and extermination camp by Gutarowska *et al.* (2015), employing metabolomics techniques to determine the metabolic products present in biofilms on brick and wooden buildings at this important site.

The metabolome includes all the small metabolic products that are produced within a cell. Sophisticated chemical analytical techniques used in its study include mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared (FTIR) spectroscopy, coupled with computerized database searches (Longnecker *et al.* 2015). Metabolomic studies can be grouped into two categories: targeted, where quantitative data on a pre-defined set of compounds is obtained, and untargeted, i.e. the search for a broader range of metabolites, some of which may not be present in the known databases (for an overview of this subject see Patti *et al.* 2012). Since thousands of such compounds may be detected, the work can be limited by throughput problems (Dunn *et al.* 2012). Other major drawbacks are the lack of suitable databases and software for analysis. Costa *et al.* (2016) suggested a new computational platform for the analysis of metabolomics data that include data loading in various formats, preprocessing, univariate and multivariate data analyses, data selection and even machine learning. This is a rapidly expanding area in the field of biodeterioration of heritage items.

BIOCONSERVATION

The use of biological organisms in the treatment of degraded stone is relatively new. Biotechnology is an innovative area in the conservation of historic and artistic heritage and biotechnological approaches have only relatively recently begun to be used for the analysis of lithotrophic organisms suitable for use in conservation (Fernandes 2006). The technologies include biomineralization, biocleaning and biomimetic surfaces.

Consolidation of stone by biomineralization, in which microorganisms (most commonly bacteria) are impregnated into deteriorated stone and allowed to produce new lithic material, is a practice already used in both conservation-restoration and civil engineering. Igneous, sedimentary and metamorphic stone have different physical characteristics that will

influence the success or failure of consolidation procedures. The majority of this work has been done with limestone and concrete. Bacteria used in the process include those from the genera *Streptomyces*, *Ralstonia*, *Bradirhyzobium*, *Methylobacterium*, *Burkholderia*, *Caulobacter*, *Bacillus*, *Myxococcus* and *Xanthobacter* (Braissant 2005).

Bacterial calcium carbonate precipitation has been proposed as an environmentally and health and safety friendly biological technique to restore and consolidate ornamental calcareous stones and other architectural heritage (Fernandes 2006; Jroundi *et al.* 2014; Alberto *et al.* 2015). Under suitable conditions, most heterotrophic bacteria are able to induce $CaCO_3$ precipitation in artificial media. The metabolic pathways that produce carbonate and bicarbonate ions and increase pH favor $CaCO_3$ precipitation in the presence of free calcium (Knorre and Krumbein 2000). More can be learned about these activities and their potential use in biotechnological applications elsewhere (Dhami *et al.* 2014; Zhu and Dittrich 2016).

Three metabolic pathways related to the nitrogen cycle can increase pH by releasing ammonia into the surroundings: (1) the deamination of amino acids; (2) the dissimilatory reduction of nitrate; and (3) the degradation of urea (Perito and Mastromei 2011). The degradation of urea produces NH_3 and CO_2 that dissolve in water, forming HCO_3^- and CO_3^{2-} ions. Macromolecules present on the surface of bacteria, in the cell walls or in extracellular polymeric substance (EPS) have anionic functional groups that also play an important role in $CaCO_3$ precipitation. Cell-attached or free EPS can trap calcium ions and influence the development of the $CaCO_3$ precipitates (Braissant *et al.* 2003). However, emerging evidence suggests that EPS could also inhibit $CaCO_3$ precipitation. The final outcome may, at least partially, be reliant on the fact that the calcium binding property is dependent on pH and the physical state of the biopolymers (Braissant *et al.* 2007).

EPS occurs in different physical forms: loosely bound, tightly bound, as slime and capsular. The use of EPS biopolymers to enhance carbonate precipitation and subsequent stone consolidation appears to be dependent on the extraction method used for EPS recovery; different states of EPS are obtained depending on the type of method employed. Even if the correct type of EPS is extracted and a suitable formulation prepared, it is possible that the EPS, while enhancing $CaCO_3$ precipitation, may obstruct the pore network within the stone, altering the flux of water and dissolved ions, thereby weakening the structure. The weakening of the mineral lattice through repeated wetting and drying cycles and concurrent expansion and contraction induces damage in the material. Thus, from a technological perspective, it seems that the use of EPS or the management of microbial processes to control the precipitation of carbonate within EPS matrices still requires future research (Decho 2010).

Calcite 'biocement' produced by bacteria is compatible with calcareous stones as it mimics their natural formation process. However, it has important limitations (Rodriguez-Navarro *et al.* 2003). Biotic stone crystals are more disorderly than the abiotic ones. Plugging stone pores with biological remains and uncontrolled biofilm growth after application of biocalcifying bacteria may prevent airflow through the stone, leading to increased decay. The use of heterotrophic bacteria that do not survive on the stone after treatment, such as *M. xanthus*, may overcome the latter limitation. Rodriguez-Navarro *et al.* (2003) used this bacterium in the consolidation of calcarenite, a highly porous stone frequently employed in Spanish historic buildings. In this study, stone slabs were steam sterilized before incubation in a growth medium, a condition that could not be applied in the real world. However, the newly formed calcite and vaterite crystals were more resistant to sonication stress and were strongly adhered to the pore surfaces without any blocking or plugging, suggesting that further experiments could be worthwhile.

The migration of the bacteria in the pores determines the distribution of the newly formed material in the stone. Because most stone degradation occurs near the surface, the bacteria should ideally stay close to that surface. On the

other hand, if the bacteria do not travel deep enough into the stone, the treatment will only be superficial and this is also an important consideration. The use of this technique on stone buildings in general has been reviewed by De Belie (2016).

An alternative to the addition of calcifying microorganisms is a process called bioaugmentation, in which the indigenous microorganisms are encouraged to proliferate by adding suitable nutrients to the stone. This approach was adopted by Jroundi *et al.* (2014) on decayed gypsum plasters from the Alcázar in Guadalajara, Spain. Treatment with conventional consolidants (ethyl silicate) was compared with bioaugmentation using a bacterial growth medium. Seven successive impregnations with the conventional consolidants were necessary because of the delapidated state of the plaster. For the bioaugmentation process, the growth medium was sprayed to saturation twice each day for six successive days. Both types of treatment were successful in terms of strengthening the plasters, but the bioaugmentation treatment produced greater depth of penetration and consolidation. The strengthening effect was associated mainly with the bacterial production of metastable vaterite. The authors later successfully used this technique *in situ* on limestone cresting on the roof of the Royal Chapel in Granada, Spain (Jroundi *et al.* 2015). The challenges of such treatments are to encourage growth of only the calcinogenic bacteria and ensure that they remain dominant in the stone over time.

Non-biologically produced calcium nanoparticles can also be used. A commercially available solution of $Ca(OH)_2$ nanoparticles has been shown to be effective in consolidating powdering painted layers of Mesoamerican wall paintings in Ixcaquixtla and Calakmul, Mexico (Baglioni *et al.* 2014). However, there can be problems associated with the carrier system: alcohol is generally recommended, but may result in localization of the consolidant too near the surface of the stone, an undesired outcome when mass consolidation is required (Borsoi *et al.* 2016).

Until recently, it was not known how the bioaugmented stone would react to the biodeteriogenic processes in the environment. Shirakawa *et al.* (2015) demonstrated that *B. sphaericus*-biocalcified fiber cement was sufficiently resistant to subsequent colonization in the subtropical environment of São Paulo, Brazil, over 22 months. The authors showed that this technology could be used not only to repair, but also to reduce the fouling-associated darkening of fiber cement. In all cases, regulatory barriers may be an impediment to the routine use of such new technologies. Regulations can vary from country to country and obtaining consent from officials usually involves considerable time and effort. This will obviously change as the technology becomes more accepted.

Lopez-Moreno *et al.* (2014) isolated $CaCO_3$-precipitating bacteria from ignimbrite stone (a siliceous stone containing small quantities of Ca) from the roof of Morelia Cathedral in Mexico, and suggested their possible use in bioconsolidation of that stone. They pointed out that EPS-producing bacteria in biofilms may be either advantageous or disadvantageous for the integrity of the stone, and that the final result from using these bacteria in bioconsolidation will vary depending on the type of stone. This has important implications for microbiological applications in stone consolidation. More recently, Tang *et al.* (2016) proposed a biological (living eukaryotic cells) autocementation kit called Euk.cement, based on the ability of silica-binding peptides on the surface of the dimorphic yeast *Yarrowia lipolytica* to immobilize onto silica particles. The yeast is modified by the integration of genes for calcium precipitation. The silica-immobilized recombinant cells then produce carbonate that cements the particles together.

Biocleaning systems utilize microbial activites, either extracted from or still within the living cells. Biocleaning aims to remove altered stone forms such as sulfate and nitrate crusts, organic substances like animal glue, and foreign materials such as graffiti pigments. Delivery systems may include sepiolite, Hydrobiogel-97, cotton, wool, Carbogel, mortar and alginate beads, agar and arbocel (Bosch-Roig *et al.* 2015). The safety of this methodology was discussed in 2014 by Bosch-Roig and Ranalli, who stressed

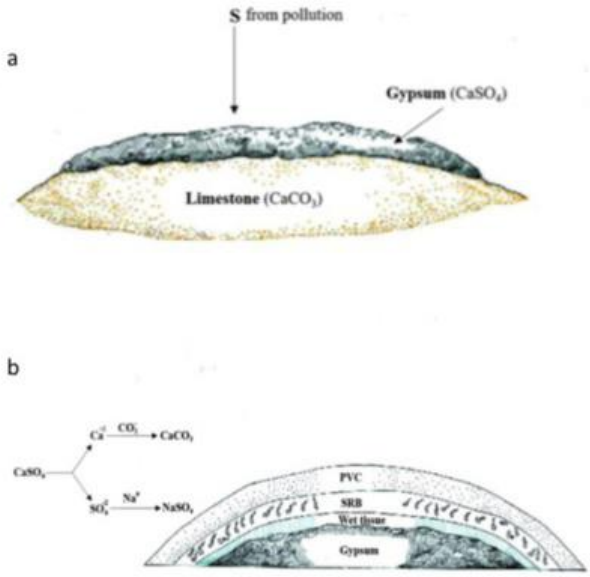

Figure 1 (a) Black gypsum formed on the surface of a limestone building by reaction with air pollutants. For the biocleaning treatment, the surface is covered with a moist tissue, followed by a carrier gel containing the aerotolerant sulfate-reducing bacterial cells (*Desulfovibrio vulgaris* subsp. vulgaris ATCC 29579) and a small amount of lactate carbon source. (b) The whole surface is then covered with a PVC film to help preserve humidity and reduce oxygen ingress. The overall reaction, changing black gypsum to white calcite and sodium sulfate, is shown on the left.

the importance of developing suitable strategies for inspection and monitoring of biocleaned buildings. Martino *et al.* (2015) used sulfate-reducing bacteria to remove the gypsum black layer deposits from the surface of a limestone polychrome bas-relief in a gallery in Palermo, Sicily (Fig. 1).

X-ray fluorescence (XRF) and FTIR investigations showed that the application of live *Desulfovibrio vulgaris* in gel form efficiently and selectively removed the sulfate deposits from large areas of the surface. Three applications of the bacteria in a Carbogel carrier were necessary to achieve the desired level of cleaning. Similar technology has been applied successfully on other important cultural monuments, including the sculpture *Allegoria della Morte* (Gioventù and Lorenzi 2013) and a tile from Milan Cathedral (Cappitelli *et al.* 2007).

Sanmartín *et al.* (2015) identified various bacterial genera and a fungal *Alternaria* sp. as candidates for removing graffiti from disfigured buildings by using painted glass slides as test substrates. Microorganisms were isolated from graffiti, an acrylic wall painting, a car in a scrapyard and the soil beneath it, and the interior of spray paint cans.

Other examples of successful biocleaning treatments include the use of *Thiobacillus* sp. to remove lichens (De Muynck *et al.* 2010), the application of enzyme extracts to whiten marble (Konkol *et al.* 2009), and the use of viable cells of *Pseudomonas stutzeri* for cleaning medieval frescoes (Lustrato *et al.* 2012). Troiano *et al.* (2013) successfully used a combination of chemical and microbiological treatments (application of *D. vulgaris* subsp. *vulgaris* ATCC 29579 after pre-treatment with the non-ionic detergent Tween 20) in the cleaning of stone. The considerable development required to produce a commercially viable biocleaning treatment is discussed by Balloi *et al.* (2017).

Biomimetic surfaces, which are protective films inspired by biologically produced

Table 1 A review of some methods used for removal or prevention of biofilms on stone.

Method	Prevention (P) or Treatment (T)	Main advantages	Main disadvantages	References
Physical				
Water jet	T	Simple, cheap	Potentially damaging, water retention is a problem Efficacy unproven Short-lived	Blasquez *et al*. 2000; Shirakawa *et al*. 2012
Irradiation:				
Visible light	T	Simple, cheap		Sanmartín *et al*. 2017;
UV	T	Simple, cheap	Unproven, ineffective against pigmented fungi	Borderie *et al*. 2014
Gamma	T	High penetration		Boniek *et al*. 2017
Laser	T	Versatile	Not always successful	Mascalchi *et al*. 2015; Senesi *et al*. 2016; Osticioli *et al*. 2015; Sanz *et al*. 2015, 2017
Chemical				
Liquid/gaseous biocides	T	Many options	Ecological/health hazards, require specialist help	Allsopp *et al*. 2004
Metal nanoparticles	P	Can be compatible with consolidants Questions on ecotoxological effects	May affect stone color	Gherardi *et al*. 2017; Gómez-Ortiz *et al*. 2013; van der Werf *et al*. 2015; Pinna *et al*. 2012; Ditaranto *et al*. 2011; La Russa *et al*. 2014; Carrillo-González *et al*. 2016; Bellissima *et al*. 2014; Munafò *et al*. 2015
Biological				
Natural biocides	T	More environmentally acceptable	May lack efficacy, unproven	Pinna *et al*. 2017; Sasso *et al*. 2016; Jeong *et al*. 2017, Rotolo *et al*. 2016
Biological agents	T/P	Non-polluting, long-lived	Efficacy unproven, unknown long-term ecological effects	Szewczyk *et al*. 2006; Silva *et al*. 2015

substances or surfaces, are interesting new approaches to stone conservation. The lithic materials may have uniform size, novel crystal morphology, specific crystallographic orientation, reduced weight, mechanical robustness and unique functions, as well as compatibility with the original stone. Liu *et al.* (2006) identified an inscription-protecting film of crude CaC_2O_4 on the Lingyin Tower in Hangzhou, China, and produced an artificial conservation film of the same material to mimic this property. Another novel biomimetic nanocomposite, comprised of amorphous silica and calcium oxalate, a protective patina often found on monuments, has been efficiently synthesized at low cost by modifying tetraethoxysilane by incorporating calcium oxalate monohydrate nanoparticles into the silica matrix (Maravelaki *et al.* 2015). The new material significantly improved porous limestone performance without changing the microstructure. Water capillary absorption coefficient, the total amount of capillary absorbed water, and water vapor permeability were decreased, while indirect tensile strength was increased.

Future developments for biomimics could include protective, but non-lithic, materials. For example, the skin of the gecko has anti-wetting and self-cleaning properties (Watson *et al*. 2015). Such characteristics could usefully be mimicked for antimicrobial coatings on historic buildings. Indeed, the superhydrophobicity of the lotus leaf surface has already led to the development of water-repellent-mimicking materials used commercially in other areas. An excellent discussion of these and other self-cleaning surfaces can be found in He (2017).

MODERN METHODS FOR TREATING AND PREVENTING COLONIZATION AND RECOLONIZATION

A variety of methods employed over the years for removing or preventing biological growths on stone is summarized in Table 1. It would appear obvious that modulation of light can affect the growth and activity of phototrophs, organisms that are immensely important in biofilms on historic buildings, and a considerable amount of work has been carried out to investigate this interaction.

Moderate light intensity can inactivate Cyanobacteria by causing photoinhibition, photobleaching and photodamage to the cells through reactive oxygen species formation. The effectiveness depends not only on light intensity but also on the particular Cyanobacteria species that are present in the biofilms. Red light is most effective against species rich in phycocyanin and allophycocyanin, such as those belonging to the genera *Leptolyngbya*, *Synechocystis* and *Scytonema*, while green light is more effective against species rich in phycoerythrin, such as *Oculatella subterranea* (Hsieh *et al*. 2014). These same authors state that blue light is the least effective, since 150 μmol photons m^{-2} s^{-1} of blue light could not cause damage to biofilms produced from Cyanobacteria isolates treated with the photosensitizer D-Ala. This may be due, in part, to the fact that blue light has high energy but low penetration ability, whereas red light has low energy but high penetration ability.

Sanmartín *et al.* (2017), examining the possibility of using colored cellophane films to control growth and color of phototrophic biofilms in bioreactors, found that blue cellophane led to complete growth inhibition of an algal and cyanobacterial mixed culture derived from a natural biofilm on a granitic building in Spain. High light levels cause photoinhibition and radical formation, reducing phototroph growth, while dim light may promote photosynthesis (Hsieh *et al.* 2013). Therefore, the apparent paradox could be explained by the intensity, rather than the quality, of light. In the Sanmartín experiment, the quantity of blue light beneath the cellophane covers, expressed as photosynthetic photon flux density, was 12.60 μmol photons m^{-2} s^{-1}, suggesting that light intensity may play a role. In a word of warning, Urzì *et al.* (2014) pointed out that treatment with certain visible light wavelengths (blue light in the aforementioned case) may actually cause an increase in the growth of certain microorganisms that can be just as, or even more, damaging.

UV-C irradiation has been shown to have deleterious effects on the growth of mixed unicellular algae isolated from caves in the Dordogne, France (Borderie *et al.* 2011). This has been suggested for controlling algal blooms in water bodies (Tao *et al.* 2010); UV-C irradiation at 20–200 mJ cm^{-2} suppressed *M. aeruginosa* growth for 3–13 d in a dose-dependent manner and UV-C irradiation at 100 and 200 mJ cm^{-2} induced severe cell disintegration in more than 70% of the irradiated cells. In a show cave in Northeastern France, however, UV-C was used against terrestrial biofilms with alternating periods of 30 min on/15 min off over 12 h at night (effective dose 180 kJ m^{-2}). Although effective after one year, recolonization occurred after 16 months (Borderie *et al.* 2014).

Gamma irradiation has often been used for the disinfection of historic materials such as documents. This was tested for the first time as an application on stone cultural property by Boniek *et al.* (2017). The researchers treated soapstone blocks that had been exposed for 12 months to the humid environment of Minas Gerais, Brazil, with 6.2 Gy/h γ-radiation for 97 minutes at a 10-cm distance. Fungi isolated from the blocks before irradiation were of the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Calcarisporium*, *Cladosporium*, *Curvularia*, *Epicoccum*, *Fusarium and Penicillium*. Of these, only *Cladosporium cladosporioides* survived and increased in number after irradiation. The authors emphasized that, although γ-radiation could be used to treat fungal colonization on stone surfaces, it would have serious limitations when the highly disfiguring, melanin-producing species are present.

The use of lasers has become common in stone conservation (Salimbeni *et al.* 2000; Bromblet *et al.* 2003). The ablation processes can utilize various wavelengths and pulse widths, giving versatility for a variety of conservation problems. Mascalchi *et al.* (2015 and references therein) used selective microwaves which proved effective and suggested that the treatment alone might be suitable to avoid future recolonization.

Senesi *et al.* (2016) used laser cleaning combined with laser-induced breakdown spectroscopy (LIBS) on the breakdown products of black crusts on a limestone castle in Italy in order to remove crusts only down to the appropriate depth. Recently, a Q-switched (QS) Nd:YAG laser operating at 532 nm successfully removed *Verrucaria nigrescens* growths from Carrara marble artefacts (Osticioli *et al.* 2015). Both fungal and algal cells were destroyed within about 100–200 μm of the surface. Other studies, however, have suggested that laser treatment is only partially successful in the removal of lichens from stone surfaces (Sanz *et al.* 2015, 2017).

Aesthetic disfiguration of stone is often related to the pigments produced by algae and Cyanobacteria (chlorophylls and carotenoids), fungi and Actinomycetes (melanins, carotenoids and others). Pigment inhibitors interfere with the production of physiologically important pigments, rendering organisms more vulnerable to environmental stress, but also reducing the disfiguring effects of microbial growth. Tricyclazole, arbutin and carpropamid are compounds that can disturb the formation of melanins or other dark pigments, while cerulenin is a potent inhibitor of carotenoid synthesis in bacteria. Although tricyclazole was shown to increase the sensitivity of the fungus *C. cladosporoides* to biocides, the effect in the field was erratic (Young *et al.* 2009). It appears that this approach has not yielded sufficiently promising results for further research.

Nanoparticles are appearing more frequently in the list of potential treatments for cultural heritage conservation. In an antimicrobial nanocoating, at least one dimension of the particle must be in the range of 10^{-9} m. One of the advantages of using nanoparticles is that stable suspensions can be achieved without the use of surfactants, which might leave unwanted residues on the stone surface (Baglioni *et al.* 2015). The suspensions can easily be brushed or sprayed onto the surface. They may also be applied as a gel, but this may have the disadvantage of leaving chemical residues that can be used by microorganisms for growth.

The antimicrobial activity of titanium dioxide (anatase) nanoparticles is well known and it is probably the most widely used

nanocoating. The biocidal efficiency of TiO_2-based engineered nanoparticles, as well as their de-polluting and self-cleaning activities, has been reviewed by Munafò *et al.* (2015). Titanium dioxide (TiO_2) spiked with various metals was used by La Russa *et al.* (2014) for testing on cultural heritage materials. They incorporated silver, iron and strontium into TiO_2 gels and tested them *in vitro* against bacteria isolated from deteriorated surfaces: *P. stutzeri*, *Stenotrophomonas maltophilia*, *Micrococcus luteus* and *B. subtilis.* As expected, the addition of iron caused the greatest chromatic change, with silver and strontium inducing merely a grey discoloration, which was more notable at higher TiO_2 concentrations. Silver, with or without the other metals, showed the greatest antibacterial activity, and could be further investigated.

Silver nanoparticles were shown to be effective protectants of gypsum (Shirakawa *et al.* 2013) and they have also been studied by application onto Italian Serena sandstone surfaces (Bellissima *et al.* 2014). In the latter case, the nanoparticles were functionalized by condensing with a silane precursor (tetraethylorthosilicate), resulting in a 50–80% reduction in cell viability. Carrillo-González *et al.* (2016), using two different plant extracts for nanoparticle synthesis, determined that silver nanoparticles were effective antimicrobial agents for application on stucco, basalt and calcite conservation, but their efficacy was dose-dependent. The authors used isolates from the walls of buildings in the important archaeological site of Teotihuacan, Mexico. The isolates were first tested *in vitro* with subsequent evaluation of the nanoparticles' efficacy against the most sensitive and rapidly growing isolates, *P. carotovorum* and *A. alternata*, which were inoculated onto stucco, calcite and basalt blocks.

Li *et al.* (2017) demonstrated that silver ions could be more effective than silver nanoparticles, at least against bacteria grown in suspension in microtiter plates. Modified titanium and silver nanoparticles were compared by Aflori *et al.* (2013), who prepared the silsesquioxane-based hybrid nanocomposites with methacrylate units containing TiO_2 and/or silver nanoparticles from titanium isopropoxide and/or 3-(trimethoxysilyl) propyl methacrylate precursors. This preparation was done in the presence of silver nitrate and a primary amine surfactant dodecylamine to avoid fissuring of the hybrid gels while drying inside the stone. The nanocomposites containing TiO_2 showed higher activity against the tested *E. coli* and *C. albicans*.

Several authors have reported on the use of polymer matrices containing copper nanoparticles for the protection of stone heritage. Ditaranto *et al.* (2011) investigated the use of copper (Cu) nanoparticles dispersed in the consolidant and water-repellent Estel 1100 for the protection of several types of calcarious stone. They demonstrated that copper was released at the surface of the treated stones, a necessary step for the inhibition of microorganisms, and that, at low nanoparticle concentrations, there were no chromatic changes other than those that normally occur with Estel 1100. Another study reported that copper nanoparticles plus a water repellent were able to prevent recolonization of sandstone, marble and plaster by crustose and foliose lichens in the archaeological area of Fiesole, Italy, over a three-year period (Pinna *et al.* 2012).

Zinc oxide (ZnO) may be more appropriate for the conservation of light colored stones. Van der Werf *et al.* (2015) demonstrated that higher levels of ZnO nanoparticles could be used without affecting the color of the stone. Using a 12th-century church in Manfredonia, southern Italy, as the test site, they showed that a tenfold greater concentration of ZnO nanoparticles than Cu nanoparticles could be utilized without affecting the color of the stone substrate. As the nanoparticles are whitish and the carriers used, Silo 111 and Estel 1000, are colorless, this would be expected. In fact, the addition of the ZnO nanoparticles reduced the slight chromatic changes that are normally observed when using consolidants/water repellents alone.

Gómez-Ortíz *et al.* (2013) developed a treatment using $Ca(OH)_2$ mixed with ZnO and TiO_2 nanoparticles, all of which were synthesized in the laboratory. The ZnO-based systems showed the best antifungal activity against *P. oxalicum*

and *A. niger* biofilms on glass slides and limestone coupons under simulated photoperiod conditions. Ti nanoparticles have also been shown to be compatible with both stone consolidants and organosiloxanes, thus allowing these traditional conservation tools to be combined with a photocatalytic cleaning component.

Gherardi *et al.* (2017) illustrated that the photoactivity range of TiO_2 could be extended to solar irradiation due to the presence of residual benzyl alcohol groups from its non-aqueous synthesis that allowed it to be combined with commercial organosiloxane consolidants. They emphasized the importance of field testing potential new treatments and designed a protocol for such trials, involving colorimetric and water absorption tests, optical microscopy, spectrophotometric measurements, and environmental scanning electron microscopy combined with energy dispersive X-ray analysis (ESEM-EDX). The two types of marble used in their tests (Candoglia and Crevoladossola) produced very different results, indicating the need to use the appropriate substrate in such testing.

Scott Mitchell and colleagues at the Spanish National Research Council in Spain perform ongoing investigations into surface treatments with liquids or gels constructed from polyoxometalates and quaternary ammonium cations. The superhydrophobic ionic liquids are composed of long-chain alkylammonium cations, of well-known antimicrobial activity, and polyoxometalate anions. These nanohybrid materials are being tested for activity on stone surfaces.

Synthetic biocides have been used widely by the cultural heritage community of conservators, restorers and biodeterioration scientists. Biocides vary in their ability to kill microorganisms, provide long-lasting effects and in their impact on stone materials. Their effectiveness in controlling microbial growth is related to the susceptibility of microorganisms and the surrounding environment. For instance, water-soluble compounds in biocidal formulations may be leached from treated surfaces by rain and run-off water. This could be particularly important in the case of buildings located in tropical zones with heavy rains (Ortega-Morales *et al.* 1999).

Those working in cultural heritage seek biocide formulations which are harmless for the users and have low environmental impact. This has prompted the search for natural molecules to develop novel, long-lasting, environmentally acceptable biocides. Emerging areas of research include the use of eco-friendly compounds derived from natural sources, such as plant metabolites (Jeong *et al.* 2017) and antimicrobial products of microbial origin. Isothyocyanates from plant sources have proved useful to prevent biofilm growth (Saravia and Gaylarde 1998) and other natural metabolites have also demonstrated their value in biofilm control in the laboratory. Sasso *et al.* (2016) found that a cell extract from *Trichoderma harzianum*, rich in a range of chemical compounds, was effective against the photosynthetic organisms *Chlorella*, *Stichococcus*, *Leptolyngbya* and *Pleurocapsa*, as confirmed by confocal laser scanning microscopy (CLSM) and chlorophyll a determinations. Antifouling compounds sourced from the sea (e.g. zosteric acid) and even lichen-associated metabolites also seem promising (Pinna *et al.* 2018 and references therein). However, it appears unlikely that such novel products will be manufactured in the near future (Pinna *et al.* 2018). However, novel treatments to eliminate and prevent biofilm colonization of stone substrates may be produced by using mixed formulations. These include the use of pigment and/or exopolysaccharide inhibitors and permeabilizers in order to increase the effectiveness of existing biocides, natural antimicrobial compounds or extracts (Young *et al.* 2009).

CONCLUSIONS

Microbial biodeterioration of built cultural heritage presents a significant problem for conservation scientists. The advent of novel technologies such as next-generation sequencing allows scientists and conservators to deal with minute amounts of material, enables the detection of specific microbial populations

such as halophiles and has also contributed to a better understanding of the complexity of microbial communities. This technology has its drawbacks, however, and improvements are necessary. A proper assessment of the biodeterioration potential of microbial deteriogens is mandatory.

Most studies rely on culture-based methods that allow the detection and characterization of deteriorating microbial metabolites. This approach has limited applicability under real-life conditions. Metabolomic profiling on environmental samples in the study of biodeterioration of building materials using ultra-high performance liquid chromatography with high-resolution mass spectrometry (UPLC-HRMS) circumvents this problem and can certainly be expected to expand in the future.

An increasing number of approaches and types of materials for the conservation of stone cultural heritage have also been discussed. Some of these approaches and materials appear to be their infancy (e.g. biomimetic surfaces and natural antimicrobials), while others, such as nanocoatings, would seem to have reached maturity, particularly for consolidation and self-cleaning purposes. An increasing avenue of evidence shows that engineered nanoparticles could also be used to control microbial growth on stone; however, their ecotoxicological risks are still to be determined. Whether some of these approaches will find real-life application depends on a range of factors. The most important drive for ultimate use is their acceptance by the conservation community. Conservators may prefer approaches and materials that originate from biological sources (natural consolidants/protectants), are inspired by nature or supported by biological processes (biocarbonatogenesis), or have a low impact on the environment (natural antimicrobials vs synthetic biocides). Such new technologies need to be validated in the field.

The variety of approaches testifies to the growth of this sector. Biodeterioration scientists need to keep in mind that the main goal of conservators is to prolong the life of cultural heritage property using effective and, if possible, simple, inexpensive materials and methods. On the other hand, conservators must assess the scientific evidence generated by biodeterioration scientists and determine its value using cost-benefit analysis and the goals of their particular institutions. Multidisciplinary teams will certainly be involved in future developments.

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