Chapter 13
Antimicrobial Properties of Nanomaterials Used to Control Microbial Colonization of Stone Substrata

B.O. Ortega-Morales, M.M. Reyes-Estebanez, C.C. Gaylarde, J.C. Camacho-Chab, P. Sanmartín, M.J. Chan-Bacab, C.A. Granados-Echegoyen, and J.E. Pereañez-Sacarias

13.1 Introduction

Subaerial biofilms are microbial communities that colonize solid surfaces exposed to the atmosphere [1]. Subaerial biofilms associated with rock substratum colonize surfaces as epilithic growths and as endolithic communities grow within fissures, cracks, and pores in the rock matrices. The extent to which surfaces are colonized by microbes depends on climatic parameters (e.g., temperature, light, and humidity), the composition of the mineral substrate, and its intrinsic properties.

The concept of bioreceptivity arose from a joint consideration of these factors. Guillitte [2] first introduced this concept, describing it as "the ability of a material to be colonized by living organisms," which does not necessarily imply biodegradation of the material. The term bioreceptivity encompasses the properties of a material that contributes to its colonization by the development of microorganisms.

B.O. Ortega-Morales (✉) • M.M. Reyes-Estebanez • J.C. Camacho-Chab
M.J. Chan-Bacab • J.E. Pereañez-Sacarias
Departamento de Microbiología Ambiental y Biotecnología (DEMAB),
Universidad Autónoma de Campeche, San Francisco de Campeche, Mexico
e-mail: beortega@uacam.mx

C.C. Gaylarde
Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK, USA

P. Sanmartín
Departamento de Edafoloxía e Química Agrícola, Facultade de Farmacia,
Universidade de Santiago de Compostela, Santiago de Compostela, Spain

C.A. Granados-Echegoyen
Centro de Desarrollo Sustentable y Aprovechamiento de la Vida Silvestre (CEDESU),
Universidad Autónoma de Campeche, San Francisco de Campeche, Mexico

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Various studies have been carried out with the aim of establishing the properties that generally affect bioreceptivity, as not all materials are susceptible to colonization by the same agents. Although not all authors agree about which factor has the greatest influence [3], it is generally considered that the most important properties affecting bioreceptivity are the chemical and mineral composition of the rock, the porosity, and the roughness of the rock surface. Several types of bioreceptivity can be defined according to the state of the material under study [2, 3]. Primary bioreceptivity indicates the initial potential of the material to be colonized. It refers to the intrinsic potential of the unaltered recently extracted or newly produced material. Secondary bioreceptivity is observed over time when a material has already been altered by the action of external factors (e.g., salts, thermal alteration, biological colonization, etc.). Tertiary bioreceptivity is defined as the type of bioreceptivity created when the material is modified by human activity such as conservation practices.

13.2 Control of Microbial Biodeterioration and Nanoscience Applied to Built Cultural Heritage

It is now accepted that microorganisms may contribute to the aesthetic and physical deterioration of stone monuments and statues. Nevertheless, recent research shows that the association between biofilms and stone weathering is not necessarily detrimental. Some studies point toward a negligible effect of biofilms or even a protective role on the underlying material [4]. Once an appropriate assessment has indicated that microbial biodeterioration is taking place, it is a common practice to implement a set of strategies that include mechanical removal, biophysical eradication, and chemical control of biofilms, either alone or in combination. The application of biocides to control microbial colonization on the stone is by far the most widespread approach and dominates in the literature [5].

Nanoparticles (NPs) have recently received attention from the conservation and restoration communities as alternatives to biocides. Nanomaterials have both photocatalytic and catalytic properties, which were first used for the consolidation of degraded stone or to enhance the properties of painted walls. According to Baglioni and co-workers, the first time that nanoscience was applied in the field of cultural heritage conservation dates back to the end of 1990 when calcium hydroxide nanoparticles were used to replace old polymer resins covering mural painting [6]. Both photocatalytic and catalytic properties help to remove dirt and polluting agents from heritage surfaces and are thus attractive self-cleaning and de-polluting agents; these properties may also include biocidal activity, as in the case of titanium dioxide (TiO_2) [7].

This contribution briefly reviews the antimicrobial properties of NPs, discusses the limitations and advantages of NP-based treatments, and highlights the importance of choosing appropriate model organisms for testing and community-based approaches.

13.3 Antimicrobial Properties of NPs Used on Stone Substrate

13.3.1 Fundamentals of Antimicrobial Activity of NPs

NPs provide an alternative to antibiotics to control gram-positive and gram-negative bacteria. The mode of action of these nanomaterials includes induction of oxidative stress, the release of metal ions, and nonoxidative mechanisms; processes can occur simultaneously. The major processes responsible for the antibacterial effects displayed by NPs include interactions with DNA and proteins, as well as penetration of cell membrane [8].

These processes are sequentially triggered, usually starting when NPs bind electrostatically to the cell wall and membranes, altering the function of membrane, leading to depolarization and loss of integrity, which causes problems in transport, energy transduction and, finally, cell death. Disruption of the respiratory chain induces a burst of ROS causing damage to macromolecules involving lipid peroxidation, enzyme inhibition, and alterations to RNA and DNA (Fig. 13.1). In some cases, the toxicity of the nanoparticles is induced by visible or UV light (photocatalytic effect) [9], which also generates ROS. Other effects of NPs include induction of nitrogen reactive species (NRS) and programmed cell death [10, 11].

Ag NPs have shown antimicrobial effects against bacteria and yeasts species. Relatively, Ag NPs have a low toxicity in our cells when compared to other metals [10]. The size and shape of the NPs influence their ability to interact with the bacte-

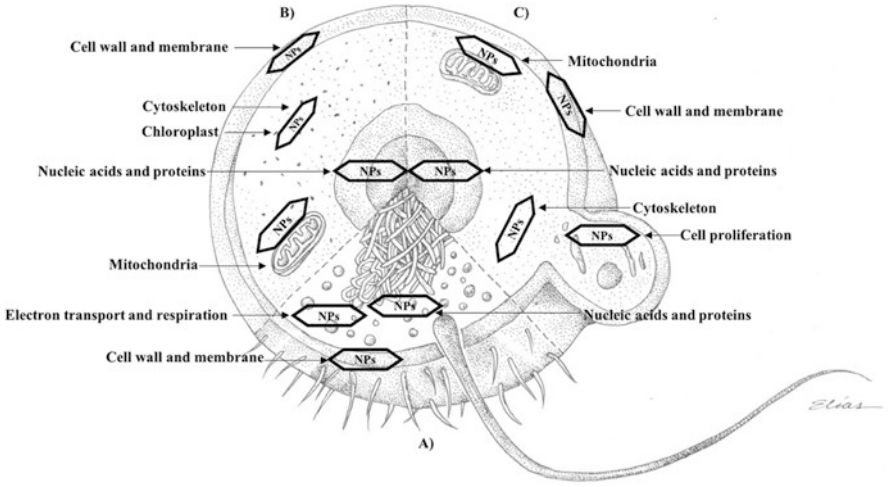

Fig. 13.1 Hypothetical model microbial cell. (**a**) Bacterium, (**b**) algae, and (**c**) fungi-yeast, depicting cellular components interacting with NPs

rial surface and release of Ag^+ into solution. Spherical NPs (1–10 nm) can effectively attach to the cell membrane's surface and disrupt permeability and respiration [12].

Ag NPs with positive charge attach to the bacterial membrane by electrostatic interactions, while negatively charged attach to P- and S-containing molecules. Ag ions are then released into the cell and inhibit respiratory enzymes, which promoted the generation of ROS and consequently damage of the cell membrane, disruption of membrane morphology increases, and permeability, leading to leaking of the internal components and resulting in cell death [13]. In order to avoid this, Ag may be complexed inside the cells, or the permeability of Ag may be reduced by combining with a mechanism to pump Ag out of the cell [13]. Nano-silver ions are also the centers of catalytic activity to activate oxygen in air or water, leading to the production of ROS, which causes lipid peroxidation [9] and also prevents the bacterial growth or kills those [8].

In the case of TiO_2 NPs, UV- or visible-light-activated TiO_2 catalyzes the cleavage of water into hydrogen and oxygen and produces ROS in solution. ROS generated through photoactivation were suggested to be responsible for the TiO_2 antimicrobial efficiency. ROS damage the cell membrane and disrupt essential membrane-bound proteins, in addition to creating single-stranded or double-stranded breaks in DNA, rendering it incapable of replication [12].

On bacterial surfaces, Cu NPs bind, as they have a high affinity for constitutive amine and carboxyl groups, causing the release of internal ions. DNA macromolecules interact with these ions that are intercalated in their chains. Since ROS can be generated both by CuO and Cu^+/Cu^{2+} ions, their effects are greater than with Ag NPs alone. In addition, Cu and CUO NPs can also induce the formation of ROS in extracellular environments. For this reason, it is considered that the released Cu ions are responsible for the microbial effect of these NPs, but the non-selectivity and toxicity to eukaryotic cells is a latent problem [13].

UV photocatalysis of ZnO NPs produces a strong bactericidal effect. Indeed, the innovation in the Zn NPs goes through the formulation of ZnO particles and the inclusion of Zn in polymers and other materials to improve their efficiency and range of action [13]. Hydroxyl radicals are generated at the surface of excited ZnO, when electrons are released from the water molecule and/or the hydroxide ions. These electrons can reduce to O_2 producing the superoxide anion. These ROS cause damage to membranes, DNA by strand breakage or oxidized nucleotides, as well as oxidation of protein catalytic centers. They can also cause external damage, as these ROS, given their negative charge, cannot pass through the membrane. They can be combined, however, with H^+, creating hydrogen peroxide, which can penetrate the membrane, causing internal damage and cell death. In addition, ZNO NPs also can generate ROS and bactericidal properties in the complete absence of light [13].

13.3.2 Antimicrobial Activity of TiO_2 NPs

TiO_2 NPs are widely used to inhibit microbial growth because of their broad-spectrum biocidal activity, and they have been the subject of considerable research on stone cultural heritage science (Table 13.1). The biocidal efficiency of TiO_2 NPs has been recently studied and partially reviewed by Batista and Munafó [14].

The nanometric form of TiO_2 is probably the most commonly used nanomaterial. Antimicrobial activity of TiO_2-based coatings has been demonstrated using a range of microorganisms, testing methods, experimental conditions, and time of trial [7] (Table 13.1). As stated previously, the biocidal effect of TiO_2 is seen under UV irradiation, causing the formation of ROS, and thus oxidative stress that attacks membrane lipids, producing damage to the membrane or DNA level [15]. The microbial groups most studied as monospecies cultures are fungi. For example, TiO_2 has been evaluated against *Aspergillus niger* (*A. niger*) or, as a mixed ZnO/TiO_2 formulation, on calcareous stone (limestone and marble coupons) [16, 17]. Both studies revealed good antifungal properties against *A. niger*, also delaying the onset of recolonization [17] or quantitatively reducing fungal coverage [16]. It is difficult to compare the efficiency of treatments since the conditions of exposure and methods of assessment varied, but it could be speculated that the synergistic effect of ZnO/TiO_2 would make this formulation more efficient and more versatile. Goméz-Ortiz [16] showed that TiO_2 was only effective under light conditions, while the mixed formulations were also effective in the dark, suggesting their potential application for indoor non-illuminated settings. Antimicrobial activity has also been ascribed to ZnO; coated nanosystems based on $Ca(OH)_2$–50% ZnO and pure zincite nanoparticulate films were effective against microorganisms, indicating that Zn NPs alone could be promising. An interesting finding of both studies is that the porosity of the substratum played a role in efficiency; more compact material performed better, presumably by allowing greater contact of NPs with fungal cells which are able to penetrate porous rock more readily [16, 17].

Phototrophs are also affected by TiO_2-based coatings. In a reported study on the biocidal effect of mixed formulation platinum-loaded titanium oxide (TiO_2/Pt) on microbial communities by Matsunaga et al. [18], the bacteria *Lactobacillus acidophilus* (*L. acidophilus*) and *Escherichia coli* (*E. coli*), the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), and the alga *Chlorella vulgaris* (*C. vulgaris*) were evaluated as individual cultures. Synergism was sought by mixing TiO_2 with platinum (Pt) loaded to increase the photoelectrochemical reaction. The approach proved valuable as in a short time (60–120 min), cell survival was reduced for all organisms, except for the alga *C. vulgaris*. This was in agreement with the previous work that found TiO_2 NPs particularly effective against gram-positive and gram-negative bacteria [19]. Further confirmation comes from another laboratory-based experiment that showed the ability of these nanomaterials to prevent fouling by the gram-negative bacterium *Stenotrophomonas maltophilia* (*S. maltophilia*) and the gram-positive *Micrococcus luteus* (*M. luteus*), each deposited individually on marble samples under conditions representing an underwater environment. There was equal activity against both bacteria, especially in the first stages of biofilm formation [20].

Table 13.1 Biocidal treatments using TiO_2 NPs to inhibit biological growth on stone materials

Substrate	Organisms involved	Type of NPs	Testing conditions (time and exposure)	Testing methods	Reference
Marble and limestone	*Aspergillus niger*	TiO_2 anatase	Outdoors, 8 days	SEM, CAM, C	[18]
Clay brick	*Chlorella mirabilis, Chroococcidiopsis fissurarum*	TiO_2	Water runoff test in laboratory conditions 10 weeks	C, DIA, CSLM	[39]
Limestone	*Aspergillus niger*, *Penicillium oxalicum* tested individually	ZnO/TiO_2	Laboratory conditions; 21 days	MCT, SEM, XRD	[17]
Mortar	*Lactobacillus acidophilus*, *Escherichia coli*, *Saccharomyces cerevisiae*, *and Chlorella vulgaris*, tested individually	TiO_2/Pt	Laboratory conditions, 120 min	MCT, CoA-C	[19]
Cover slips	*Escherichia coli, Candida albicans*, tested individually	Methacrylate + TiO_2 or Ag NPs	Laboratory conditions 24 h	ADT	[21]
Mortar slabs (sand/cement)	*Volvox*, *Chlorella*, *Aphanothece*, and *Pleurococcus*, bacteria and protozoa community	Silane, polyhydroxymethylsiloxane, emulsifier POE, and TiO anatase or ZnO emulsions	In laboratory conditions, antifouling rig setup for 8 weeks	ACI, WCA, C, Ph-S	[43]
Stone	*Stenotrophomonas maltophilia* and *Micrococcus luteus*	TiO_2, Ag, Fe, Sr alone, and mixed TiO_2 + Ag, Fe, Sr; TiO_2 + Ag + Sr, TiO_2 + Fe + Sr, TiO_2 + Ag + Fe	In vitro diffusion test on agar	ADT, CTM	[22]
Brick substrata	*Chlorella mirabilis*, *Chroococcidiopsis fissurarum*	TiO_2 + Cu, TiO_2 + Ag	Accelerated water runoff test, 11 weeks	C, MM	[1]
Travertine	*Chlorella* sp., *Klebsormidium* sp., *Phormidium* sp., *Chlorogloeopsis* sp. mixture	TiO_2, TiO_2 + Ag NPs, TiO_2 + Cu NPs	Accelerated water runoff test, 9 weeks	C, S, SEM-EDX, DIA	[15]

Plaster	Bacteria (*Bacillus paralicheniformis*, *Rhodococcus trifolii*, *Microbacterium chocolatum*, *M. kitamiense*, *Nesterenkonia sandarakina*, and *Parapedobacter koreensis*) Fungi (*Ciliophora*, *Devriesia* sp., *Cladosporium* spp., *Aureobasidium pullulans*, *Cephalosporium* sp.) Phototrophs (*Chlorella* sp., *Leptolyngbya* sp., *Chlorella*-like)	Pretreatment biotin + ethanol 5% then TiO_2, TiO_2 + Ag NPs + nanosilica	In situ on wall of stone monument, 8 months	EM, SEM-EDX, MCT, MI	[23]

ACI agar culture identification, *ADT* agar diffusion test, *C* colorimetry, *CAM* contact angle measurement, *CoA-C* acetyl coenzyme A concentration, *CSLM* confocal scanning laser microscopy, *CTM* contact time microscopy, *DIA* digital image analysis, *EM* epifluorescence microscopy, *GC-MS* gas chromatography-mass spectrometry, *MCT* microbial culture techniques, *MI* molecular identification, *MM* mathematical model, *PhS* photospectroscopy, *S* spectrophotometry, *SEM* scanning electron microscopy, *SEM-EDX* scanning electron microscopy, energy-dispersive X-ray spectroscopy, *WCA* water contact angle, *XRD* X-ray diffraction

Aflori et al. (2013) [21] tested the ability of nanomaterials containing Ag NPs with or without TiO_2 to prevent colonization of substrates by the bacterium *Escherichia coli* (*E. coli*) and the fungus *Candida albicans* (*C. albicans*) in laboratory conditions using cover slips. The presence of TiO_2 improved the biocidal effect of Ag NPs, even without UV illumination. The Ag NPs alone were equally effective against the bacteria and the fungus, whereas TiO_2 plus Ag was more effective in preventing fungal growth than in preventing bacterial growth.

In a more complex study in a terrestrial outdoor environment, La Russa [22] tested the biocidal action of NPs (Ag, Fe, Sr, and TiO_2) and doped metal nanocomposites (TiO_2, Ag-TiO_2, Sr-TiO_2, Fe-TiO_2, Ag-Sr-TiO_2, and Ag-Fe-TiO_2). In all cases, the presence of the metal increased the capacity of TiO_2 to absorb visible light. The most effective materials were Ag-TiO_2 and Ag-Sr-TiO_2, and the least effective was Ag-Fe-TiO_2. The authors attributed the differences in efficacy to the ionic radius of the metal targets. Strontium (Sr) and Ag have the largest radii, larger than that of the titanium to which the doping metal is adsorbed. In contrast, the radius of iron is smaller than that of titanium, and the metal is inserted deeply into the TiO_2 structure, rendering it inactive.

Few studies have been carried out under field trials and mixed species/experimental conditions. One of these was carried out by Ruffolo [23], who evaluated the effect of a nanocomposite of TiO_2 on a multispecies microbial community of bacteria, algae, and fungi over 8 months. They found that in these in situ conditions, variable results were obtained. For example, *Bacillus paralicheniformis* (*B. paralicheniformis*) was isolated in all treatments tested, while *Paracoccus caeni* (*P. caeni*) and *Microbacterium chocolatum* (*M. chocolatum*) were isolated from one sample only, and *Pseudonocardia* sp., *Rhizobium trifolii* (*R. trifolii*), *Microbacterium* sp., and *Bacillus* sp. from one other sample, even though all the samples were exposed to the same environmental conditions. The time selected for the experiment was appropriate to show that the nanocomposite was effective, retarding growth in treated areas. However, the efficiency of the nanomaterial in these high-humidity conditions is questioned, since recolonization was found to begin in the treated areas after 4 months. More long-term in situ tests on microbial communities are necessary.

Fonseca and co-workers [24] pioneered the approach of carrying out both laboratory and on-site studies. They tested the ability of pure TiO_2 anatase and iron-doped anatase (Fe-TiO_2) to prevent colonization of mortar slabs by a mixed culture of two green microalgae *Stichococcus bacillaris* (*S. bacillaris*) and *Chlorella ellipsoidea* (*C. ellipsoidea*) and a cyanobacterium *Gloeocapsa dermochroa* (*G. dermochroa*) in the laboratory and to prevent fouling by lichens and phototrophic microorganisms on walls of the Palácio Nacional da Pena (Sintra, Portugal). Both the pure and the Fe-doped TiO_2 anatases were effective, and better results were achieved than with conventional biocides.

13.3.3 Antimicrobial Activity of Ag NPs and Mixed Formulations

A variety of products exist for the conservation of stone, paper, and mortars, the active principles being based on NPs and nanoemulsions of hydroxides of calcium, magnesium, strontium, ferrite, silicon oxide, magnesium, zinc, and Ag.

Certain nanoproducts can produce a biocidal effect themselves, and these are useful for the control of the biodeterioration of stone materials. The sensitivity to Ag NPs was evaluated with the major microbial contaminants from various objects, using 32 bacterial and fungal strains [25]. The size of the silver nanoparticles Ag NPs produced under chemical synthesis was 10–100 nm, defining its effective concentration for the removal of microorganisms present on the surface of the objects at 45 ppm. The results of the experiment allowed the elimination of 94% of the microorganisms present, with the exception of *Bacillus subtilis* (*B. subtilis*) and *Staphylococcus xylosus* (*S. xylosus*) [25]. In the same year, a coating containing approximately 0.0001–0.01% dry weight of nano-sized Ag demonstrated its efficacy in laboratory (agar plate) studies on painted fiberglass panels inoculated with either *Aureobasidium pullulans* (*A. pullulans*) or a mixture of *Alternaria alternata* (*A. alternata*) and *Penicillium pinophilum* (*P. pinophilum*) [26]. A year later in a study conducted in Brazil [26], the addition of an aqueous solution of 10% nano-silver product (SOLTICIDE G-30) into preformed gypsum panels resulted in the reduction in discoloration caused by *Cladosporium* sp. [26]. Table 13.2 summarizes the biocidal activity of Ag NPs.

In another study, Shirakawa and co-workers [26] evaluated the effectivity of Ag NPs against *Cladosporium* sp. They found that the effectivity of the treatments was reached at a concentration of 5–15 ppm. The use of Ag NPs at a concentration of 2% or 3% in the silicone paint was particularly noteworthy. The greatest efficiency of the protective coating was noted in a mixed system, Ag NPs (1%) and ACTICIDE® EPW (0.1%), an aqueous phase biocide composed of a combination of benzimidazole carbamate, 2-octyl-isothiazolin-3-one (OIT), and a urea derivative; this achieved 100% inhibition of tested fungal growth [27].

Roy et al. [28] published a study on the synthesis of Ag NPs by microorganisms. The extracellular enzyme nitrate reductase, produced by the fungus *Aspergillus foetidus* (*A. foetidus*) MTCC8876, was utilized for NP production [25]. In an alternative microorganism-linked synthesis, Ag NPs were produced using the biogenic volatiles of the bacterium *Nesterenkonia halobia* (*N. halobia*); their antimicrobial activities were evaluated against the gram-positive bacterium *Streptomyces parvullus* (*S. parvullus*) and the fungus *A. niger.* The Ag NPs were mixed with two types of consolidation polymers and used to coat the external surfaces of sandstone and limestone blocks. The stones treated with silicon polymer loaded with Ag NPs showed an elevated antimicrobial potential against *A. niger* and *S. parvullus* [29]. In a similar study with fungi, the effectiveness of Ag NPs was proven with an amount of 5–15 ppm [27]. Silver NPs produced by microorganisms and another bio-method were screened against 19 fungal isolates from Meymand rocks and historic village. They were able to impact on 68% of fungal isolates [30].

Table 13.2 Biocidal treatments using Ag NPs to inhibit biological growth on stone materials

Substrate	Organisms involved	Type of NPs	Testing conditions (time and exposure)	Testing methods	Reference
Stucco, basalt, and calcite	Bacteria (23), fungi (14), *Pectobacterium carotovorum Alternaria alternata*	Ag NPs by green synthesis	Laboratory conditions 72 h, 120 days	CCP, DIA	[33]
Gypsum	*Cladosporium* sp.	Ag NPs	Laboratory conditions 5 weeks	C, SEM-EDX	[26]
Limestone	*Chlorella vulgaris*	Tetraetoxysilane +$AgNO_3$ or chitosan or hydrophobic silica	Accelerated test 4 weeks	C, F (chlorophyll)	[38]
Cover slips	*Escherichia coli*, *Candida albicans*	Methacrylate + TiO_2 or Ag NPs	In vitro, 24 h	ADT	[21]
Travertine	*Chlorella* sp., *Klebsormidium* sp., *Phormidium* sp., *Chlorogloeopsis* sp. mixture	TiO_2, TiO_2 + Ag NPs, TiO_2 + Cu NPs	Accelerated water runoff test 9 weeks	C, S, SEM-EDX, DIA	[15]
Stone	*Stenotrophomonas maltophilia* and *Micrococcus luteus*	TiO_2, Ag, Fe, Sr alone, and TiO_2 + Ag, Fe, Sr; TiO_2 + Ag + Sr; TiO_2 + Fe + Sr; TiO_2 + Ag + Fe	In vitro diffusion test on agar	ADT, CTM	[22]
Concrete	*Chlorella vulgaris*	Water repellents (stearates, silanes, biocides, 3-trimethoxy silyl propyl dimethyl octadecyl ammonium chloride, zeolite, 2,3,5,6, tetrachloro-4-methylsulfonyl-pyridine, and Ag NPs	Modular setup in laboratory conditions 2 weeks	VA, C	[37]

(continued)

Table 13.2 (continued)

Substrate	Organisms involved	Type of NPs	Testing conditions (time and exposure)	Testing methods	Reference
Plaster	Bacteria (*Bacillus paralicheniformis*, *Rhodococcus trifolii*, *Microbacterium chocolatum*, *M. kitamiense*, *Nesterenkonia sandarakina*, and *Parapedobacter koreensis*), fungi (*Ciliophora*, *Devriesia* sp., *Cladosporium* spp., *Aureobasidium pullulans*, *Cephalosporium* sp.), phototrophs (*Chlorella* sp., *Leptolyngbya* sp., *Chlorella*-like)	Pretreatment biotin + ethanol 5% then TiO_2, TiO_2 + Ag NPs + nanosilica	In situ on wall stone monument, 8 months	EM, SEM-EDX MCT, MI	[23]

CCP[*]chromatic changes photograph, *F* fluorimetry (chlorophyll A), *VA* visual assessment

Numerous studies have demonstrated that the use of plants in the synthetic process offers greater advantages over other biological processes. Plant extracts function as synthesis-inducing agents, giving the NPs increased stability and durability. Kalishwaralal et al. [31] studied the anti-biofilm activity of Ag NPs against *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus epidermidis* (*S. epidermidis*) biofilms. They demonstrated that the NPs could effectively block the synthesis of extracellular polymeric substances (EPS). Green synthesis using the aqueous fruit extract of *Aegle marmelos* (*A. marmelos*) allowed the generation of Ag NPs that have the ability to block EPS or biofilm production by the bacterial isolates [32].

Leaf aqueous extracts from two species, *Foeniculum vulgare* (*F. vulgare*) and *Tecoma stans* (*T. stans)*, were used in Ag NP synthesis [32]. These NPs were tested in situ to protect stucco, basalt, and calcite materials. Ag NPs from *F. vulgare* were more effective for in vitro microbial growth inhibition than those from *T. stans*. The use of Ag NPs as a preventive or corrective treatment decreased microbial colonization in three kinds of stone, and so it was recommended to use this class of NPs as antimicrobial agents to prevent future mechanisms of biodeterioration [33].

In a study comparing the biological resistance of green and conventional building materials before and after nano-metal treatment to improve fungal growth resistance, *Aspergillus brasiliensis* (*A. brasiliensis*) or *Penicillium funiculosum* (*P. funiculosum*) was inoculated on samples, and their growth was visually evaluated according to ASTM G21–09 [33]. Without nano-metals, green materials were not more prone to fungal growth than conventional ones. After nano-metal treatment at

the highest selected dose, the observed order of fungal growth resistance was nano-zinc = nano-copper > nano-silver for wooden flooring and green wooden flooring; nano-zinc > nano-silver = nano-copper for gypsum board; nano-zinc > nano-silver > nano-copper for gypsum board, calcium silicate board, and green calcium silicate board; nano-silver > nano-copper = nano-zinc for mineral fiber ceiling; and nano-silver > nano-copper > nano-zinc for green mineral fiber ceiling [34].

A silver-silica nanocomposite-based geopolymer antibacterial mortar has been developed by simple adsorption of silver in a suitable amount of a colloidal silica suspension [35]. The silver NPs (3–7 nm) were attached to the surface of 20–50 nm-sized silica NPs. Mechanical strength, durability, and mechanistic antibacterial activity of the silver-silica nanocomposite-modified geopolymer mortar (GMAg-Si) were investigated and compared to nanosilica-modified geopolymer mortar and control cement mortar. Inhibition and mortality of gram-positive and gram-negative bacteria were assessed in liquid cultures. At 6% (w/w), the GMAg-Si (cured at ambient temperature) showed substantial improvement in mechanical strength, durability, and antibacterial properties. ROS generation and cell wall rupture, as observed by fluorescence microscopy and field emission scanning electron microscopy (FESEM), have been suggested as reasons for the antibacterial efficacy of the GMAg-Si [35].

Three nanomaterials, (Ag, TiO_2, and CuO), at three concentrations (5, 10, 15 μg/mL), were evaluated for inhibition of bacterial and fungal growth [35]. Three fungi, *A. niger*, *A. flavus*, and *A. fumigatus*, and three gram-positive bacillary bacteria were isolated from three ancient Egyptian funeral masks. Ag NPs exhibited activity against *A. niger*, *A. flavus*, and *A. fumigatus* at 15 μg/mL. Ag NP reactivity with bacteria was higher than for fungi [36].

De Muynck et al. [37] performed one of the first studies comparing the antimicrobial properties of nanomaterials with those of conventional biocides. They applied Ag NPs to concrete materials to prevent colonization by phototrophs. However, the treatment was not as effective as expected such as proved by Eyssautier-Chuine et al. [38]. Moreover, the treated samples showed very noticeable color changes, with pronounced darkening of the surface. Graziani and D'Orazio [39] found that, in addition to UV light, the efficacy of the nanocoating was influenced by the properties of the material (roughness and porosity). They also applied a suspension of the same organisms as used in the previous study [40] to ancient bricks and found that the nanocoating was effective when combined with UV-A light, except on rough areas of the substrate. In an attempt to overcome this problem, the research group tested nanostructured solutions of TiO_2-Ag and TiO_2-Cu. MacMullen [41] tested the ability of a combination of Ag, TiO_2, and ZnO NPs in silane/siloxane emulsions applied to mortars to prevent fouling by algae (mainly *Chlorella vulgaris*), cyanobacteria (*Synechococcus*), bacteria, and protozoa, all isolated from Canoe Lake in Portsmouth (UK). The bioreceptivity of the material seemed to be reduced by the presence of Ag NPs, which also enhanced water repellent façade treatments.

Within the framework of COMAS (Consevazione in situ dei Manufatti Archeologici Sommersi) project, aimed at preventing the deterioration of underwater archaeological artifacts, Ruffolo et al. [23] tested the ability of TiO_2, ZnO, and

Ag NPs dispersed in siloxane wax to prevent fouling by algae, barnacles, bryozoans, and marine worms. They carried out laboratory and in situ studies over a period of 2 years in the underwater archaeological park of Baia (Naples, Italy). After 2 years, all treatments had reduced the colonization to 25–50% of that in untreated samples. The best result was achieved with TiO_2 mixed with Ag, while the poorest results were obtained with ZnO. The efficacy of the products was not increased by applying larger quantities. All treatments seemed to prevent colonization by endolithic species, which are more powerful degradation agents than epilithic species.

13.3.4 Antimicrobial Activity of ZnO NPs, CuO NPs, and Mixed Formulations

Cu NPs have been poorly explored for the protection and conservation of stone substrates. However, they have good antimicrobial properties either alone or together with other elements, and have the ability to generate multiple toxic effects, such as the formation of ROS, lipid peroxidation, protein oxidation, and DNA degradation, which was detected in *E. coli* as a model microorganism [42].

Cu NPs have been studied for their activity against phototrophs, especially the chlorophyte *Chlorella*, a common microalga on various stone surfaces. This microalga has been shown to be susceptible to Cu NPs, either as an individual [1] or in a phototrophic microbial community [15]. In both cases accelerated runoff tests were carried out over a reasonable period of exposure (9–11 weeks).

Other microbial groups less studied are bacteria and yeasts. Zazuela et al. [43] evaluated the activity of a CuO/SiO_2 nanocomposite to protect stone surfaces and showed a decrease in growth of *E. coli* (CECT 01) and the yeast *S. cerevisiae*. Essa and Khallaf [44] showed the effect of nanocomposites consisting of consolidant polymers (acrylates and silicon) and Cu NPs on target microorganisms such as *Aspergillus* spp., *Candida albicans*, and *Penicillium chrysogenum*, considered as cosmopolitan fungi, or the pathogens *Fusarium solani* (plants) and *C. albicans* (human). Ditaranto et al. [45] used as consolidant the water repellent commercial product (ESTEL 1100) as dispersing medium for Cu NPs. The authors inoculated *Arthrobacter histinolovorans* (ATCC 11442) and evaluated this product applied to calcareous stone and characterized the surface physical properties. The nanocoating strongly inhibited this soil bacterium. However, most microorganisms tested are not common inhabitants of stone surfaces and could provide an unrealistic response in comparison with the behavior of autochthonous microorganisms growing in stone communities. All studies on antimicrobial properties of Cu NPs on the stone substrate are based on microscopy (optical, SEM), microbial culture techniques, and colorimetry, which are enough to demonstrate a positive effect (Table 13.3). However, other interesting techniques to evaluate the efficiency of nanocomposites, especially those with water-repellent properties, include water absorption by contact sponge method (WACSN) [46], water absorption by capillarity (WAC), and

Table 13.3 Biocidal treatments using Cu NPs to inhibit biological growth on stone materials

Type of rock	Organisms involved	Type of NPs	Testing conditions (time and exposure)	Testing methods	Reference
Calcareous	*Arthrobacter histidinolovorans*	ESTEL 1100 + Cu NPs	In vitro laboratory conditions	MCT, SP, C, SEM-EDX	[45]
Sandstone, marble, plaster	*Aspicilia calcarea*, *Aspicilia contorta* ssp. *hoffmanniana*, *Caloplaca aurantia*, *Caloplaca crenularia*, *Diploicia canescens*, *Diploschistes actinostomus*, *Diplotomma ambiguum*, *Lecanora campestris*, *Lecanora pruinosa*, *Lecanora muralis*, *Parmelia loxodes*, *Parmelina tiliacea*, *Physcia adscendens*, *Tephromela atra*, *Verrucaria nigrescens*, *Xanthoria elegans*, *Xanthoria parietina*	Tetraethylorthosilicate, methylethoxy polysiloxane, Paraloid B72, tributyltin oxide, dibutyltin dilaurate, Cu NPs	In situ, 33 months	OM, C, FTIR, WACSM	[46]
Clay brick	*Chlorella mirabilis*, *Chroococcidiopsis fissurarum*	TiO_2 + Cu, TiO_2 + Ag	In laboratory conditions by water runoff test 11 weeks	C, MM	[1]
Limestone, sandstone	*Escherichia coli* Z1, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Streptomyces parvulus* and *Bacillus subtilis*, *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum*, *Fusarium solani*, *Alternaria solani*	Methyl and ethyl acrylate, silicon, silane/siloxane + $CuSO_4$, and Cu NPs	In laboratory conditions on microbial culture techniques 24 h	MCT, SEM, EDX	[44]

(continued)

Table 13.3 (continued)

Type of rock	Organisms involved	Type of NPs	Testing conditions (time and exposure)	Testing methods	Reference
Limestone	*Escherichia coli*, *Saccharomyces cerevisiae*	CuO/SiO_2	Laboratory conditions 15 days	MCT, C, SEM, WAC, WCA	[43]
Traventine	*Chlorella* sp., *Klebsormidium* sp., *Phormidium* sp., *Chlorogloeopsis* sp.	TiO_2, TiO_2 + Ag NPs, TiO_2 + Cu NPs	Accelerated runoff test 9 weeks	C, S, MCT, SEM, EDX, DIA	[15]

FTIR Fourier transform-infrared spectroscopy, *MM* mathematical model, *WAC*[5] water absorption by capillarity, *WACSM*[4] water absorption by contact sponge method

water contact angle (WCA) [8]. In situ experimentation studies are relatively rare. Pinna et al. [46] compared the performance of traditional treatments (tetraethylorthosilicate, methylethoxy polysiloxane, Paraloid B72, tributyltin oxide, dibutyltin dilaurate) and Cu NPs in the prevention of recolonization of sandstone, marble, and plaster by crustose and foliose lichens in the archaeological area of Fiesole, Italy. The changes in the bioreceptivity of treated materials were monitored over a period of almost 3 years. The Cu NPs plus water-repellent material yielded good results in terms of preventing biological colonization. In addition, a strengthener and water-repellent material to the Cu NPs also helped to prevent recolonization of the surfaces.

The antimicrobial activities of Zn NPs used individually or as nanocomposite with other agents have also received scant attention (Table 13.4). The principal biological target to test these NPs is fungi, especially those belonging to the genus *Aspergillus* and *Penicillium oxalicum* [8, 17, 20, 47–50], which are known stone inhabitants and deteriogens. The principal natural stones evaluated for protection using ZnO-NPs are calcareous [8, 23]. Using a microbial community model, Zang and co-workers [20] studied the biocidal effect of Zn and Ti nano-oxide silane/siloxane emulsions in laboratory conditions. They evaluated an alga-dominated microbial community and observed that these nanomaterials only slightly inhibited the growth of phototrophs.

13.4 Discussion

Stones are colonized by complex microbial communities [51]. Their composition depends on climate and microclimate, as well as the intrinsic properties of the substrate. Tertiary bioreceptivity is determined by the influence of any human activity that interferes with the material such as consolidation, cleaning, or antimicrobial treatment based on NPs and biocides.

Table 13.4 Biocidal treatments using Zn NPs to inhibit biological growth on stone materials

Substrate	Organisms involved	Type of NPs	Testing conditions (time and exposure)	Testing methods	Reference
Limestone	*Aspergillus niger* or *Penicillium oxalicum*	$Ca(OH)_2$.ZnO, $Ca(OH)_2$.TiO	Laboratory conditions, 28 days	MCT, SEM	[48]
Mortar slabs (sand/cement)	*Volvox*, *Chlorella*, *Aphanothece*, and *Pleurococcus*, bacteria and protozoa community	Silane, polyhydroxymethylsiloxane, emulsifier POE, and TiO anatase or ZnO emulsions	Laboratory conditions antifouling rig setup 8 weeks	MCT, WCA, C, Pn-S	[20]
Glass slides and Limestone	*Aspergillus niger* or *Penicillium oxalicum*	$Ca[(OH)_3]_2.2H_2O$	In vitro, 21 days, Monospecies assay	OM, SEM	[17]
Calcareous	*Aspergillus niger*	ESTEL 1000, 1100, SILO 111, ESTEL 1000 + ZnO, ESTEL 1100 + ZnO, SILO111 + ZnO, ESTEL 1100 + Cu, SILO III + Cu	In situ, 6 months	C, SEM-EDX, MI, MCT	[49]
Agar plate	*Alternaria alternata*, *Aspergillus niger*, *Penicillium chrysogenum*, and *P. pinophilum* mixed	ZnO	In laboratory conditions 10 days	BP, MI	[47]
Limestone, dolostone, and glass slides	*Aspergillus niger*, *Penicillium oxalicum*, *Paraconiothyrium* sp., *Pestalotiopsis maculans*	MgO, Zn, ZnO/MgO	In laboratory conditions	OM, ESEM-BSE, FESEM, DIA, C	[50]

BP biomass protein of exopolymeric substances (EPS), *ESEM-BSE* environmental backscattered electron scanning microscopy, *FESEM* field emission scanning electron microscopy, *OM* optical microscopy, *VCS* virtual crosshatching system

The use of nanomaterials to control microbial colonization has increased considerably in recent years. The nanometric form of TiO_2 is probably the most commonly used nanomaterial. TiO_2 NPs are characterized by their broad-spectrum biocidal activity and by being nontoxic, highly photoreactive, chemically stable, and inexpensive. However, the reactivity of nanoscale TiO_2 is light dependent. The biocidal activity of the material is greater under UV-A light than under visible light (natural or artificial), and the material is ineffective indoors and in closed environments with no light. In some studies, the TiO_2 matrix has been added to other metal NPs, including Ag [1, 14, 21–23], Cu [1, 8, 14, 46], and Pt [19].

Ag, Zn, and Cu NPs have also been shown to be effective in microbial control, either used as sole biocide agents or in mixed formulations. It is difficult to assess the effectiveness of treatments and perform direct comparisons between the published studies due to the heterogeneity of methods employed to test antimicrobial activity and the range of microorganisms tested. However, it appears that synergism is an important feature of the reviewed studies. The use of standard methods and model organisms would improve our understanding of their biocidal activity, specificity, and their interaction with the substrate.

The application of a nanomaterial that displays antimicrobial properties should reduce the bioreceptivity of a substrate. On the other hand, the tertiary bioreceptivity of a substrate should be lower than the primary bioreceptivity. In this respect, numerous laboratory studies have been conducted to evaluate the inhibitory efficacy of novel products over several weeks in controlled colonization tests with microbial communities. In particular, such studies have used bacteria, which tend to grow faster than algae and cyanobacteria, even though the latter is considered to be the most abundant colonizers of façades that usually appear before other species such as fungi, lichens, mosses, and other bryophytes. In situ (field) studies in this area are much less frequent. Indeed, the first report of a case study of the direct use of a TiO_2 coating biocide on cultural heritage buildings has been published very recently. Ruffolo et al. [23] conducted an 8-month-long study to monitor phototrophic and chemoorganotrophic colonization in the archaeological site of "Villa dei Papiri," Ercolano (Naples, Italy). These researchers found that TiO_2 NPs (alone or combined with Ag) enhanced the effect of the previously applied organic conventional biocide (biotin R). Four months after application of nanoproducts, no recolonization of the surfaces was observed. By the end of the study, the recolonization rate was higher in damper areas of the surface, suggesting the presence of water reduces the effectivity of the nanomaterial.

The characteristics of the target microorganisms influence the response to NPs. The most important of these characteristics are structural properties such as the complexity and thickness of the cell envelope. Thus, for example, fungi are usually less affected by TiO_2 than the structurally more complex bacteria and viruses [7], although Shirakawa et al. [26] demonstrated the efficacy of TiO_2 against fungal colonization on the modern glass in a 5-month field study in Sao Paulo, Brazil. Since the activity of antimicrobials is so dependent on the structure and activity of the target organisms, it is of maximum importance that they are tested in the laboratory against a wide range of organisms before being transferred to field trials carried out under the actual condi-

tions and on the same substrates as their intended use. Model organisms for testing need to be representative inhabitants of the stone subaerial environment, which generally have thick capsules, are highly pigmented, overcome desiccation by producing osmolytes, exhibit a mucoid phenotype, and possess low surface-to-volume ratios (reduced contact with the environment) [52, 53]. Low surface-to-volume ratio reduces biocide uptake. Similarly, thick capsules and sheaths would make it difficult for NPs to enter the cells. Displaying a mucoid phenotype, indicative of EPS production, would be valuable to microorganisms as a barrier for NP contact or even to reduce their activity. Few organisms of the reviewed studies possess these attributes. The reported studies should, therefore, be considered cautiously to extrapolate efficiency for outdoor building surfaces, as NPs may have a low efficiency.

Microbes generally prefer to colonize damp substrates. Nanomaterials formulated with the aim of preventing biofouling therefore often include hydrophobic properties in addition to biocidal and sometimes strengthening properties. The hydrophobic properties also reinforce the biocidal properties by preventing direct contact between organisms and the surface to be colonized [7]. Nanomaterials with these properties will also help protect mineral surfaces, as water is one of the main factors involved in the decay of such substrates. In planning a field trial to test such new protective treatments, the most stringent conditions should be used; hot and humid environments are preferred. Hence companies producing and testing antimicrobials generally use test sites in the humid tropics and suitable sites containing culturally important buildings abound in, for example, India, Latin America, and the Far East.

The potential of NPs in the field of conservation of cultural heritage has been established in a number of ways, consolidating decayed materials, enhancing and de-polluting surfaces, self-cleaning, or as a biocide in biodeterioration [7, 8, 53, 54]. There is limited understanding of the environmental fate of NPs after release from treated surfaces and the impact on surrounding nontarget organisms and ecological processes. Gladis et al. [55] previously stressed the importance of ecotoxicological assessments of active agents under development. The release of NPs may result when the coatings are not fixed adequately to stone or when the durability of materials is not sufficiently effective to remain adhered to the rock over a long period of time [56–59]. Using runoff experimental setups were able to demonstrate unequivocally direct release of Ag NPs and TiO_2 NPs from façade paints (aged and new coatings) and their transport into surface waters and soils. Research on risk assessment of environmental impact and human health issues associated with the release of NPs into the surrounding environment in field studies is essential [7].

13.5 Conclusion

In this chapter, the antimicrobial properties of major types of NPs (TiO_2, Ag, Cu, and Zn) and their mixed formulations that have been reported for stone conservation were reviewed. The heterogeneity of testing methods and microorganisms tested render comparisons difficult. However, some patterns arise. TiO_2 NPs are the most studied

type of NPs displaying biocidal properties. Comparatively, little is known about the potential of Cu and Zn NPs, although they appear to be particularly useful for indoor surfaces lacking light. There are no clear specific activities against microbial groups, as several NPs appear to exert toxic activity using similar modes of action. There is an overrepresentation among studies of medical or soilborne microorganisms, which are not representative of the stone subaerial environment. Also, as most studies are carried out under lab conditions using mono- or dual species (two organisms), there is not currently a solid body of knowledge on the effectiveness of NPs in field studies for use on building surfaces. Further studies need to be carried out using more complete and realistic approaches. These approaches include the use of standardized testing methods, preferably community-based techniques such as phospholipid fatty acids combined with molecular biology, a combination that provides information about biomass, diversity, and function of the communities. The use of model organisms from subaerial habitats and long-term field trials are also necessary.

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