Review

Perspectives for Antimicrobial Nanomaterials in Cultural Heritage Conservation

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SUMMARY

The biodeterioration of artistic and architectural heritage represents a serious and recurring problem for museums, local authorities and private collectors alike, where irreparable damage to unique artifacts can result in immeasurable losses to our shared cultural heritage. Here we present an overview of the current trends in antimicrobial products used to protect heritage items from microbial colonization and prevent their deterioration. From a conservation-restoration standpoint we contrast and compare traditional antimicrobial products with the state-of-the-art in antimicrobial nanomaterials applied in the heritage conservation field, highlighting the promising potential of various different nanomaterials, as well as points of concern and clear red flags from some of the emerging research. Through an examination of the growing body of research in the academic literature we offer recommendations and practical advice on selecting appropriate microbiological assays and characterization techniques to better evaluate the *in vitro* and *in situ* antimicrobial properties of nanomaterials.

Keywords: Nanoparticles, nanomaterials, antibacterial, antimicrobial, biocidal, fungicidal, cultural heritage, conservation, biodeterioration.

THE BIGGER PICTURE

Microorganisms are highly proficient at inhabiting and decaying cultural heritage objects and architectures. Unfortunately, only a limited range of physical and chemical methods are available for preventing or removing microbiological damage to heritage items where, all too often, the available methods are frequently inadequate to clean effectively and prevent recolonization. Our shared cultural heritage is an invaluable social and environmental resource as well as a key global economic driver. So how can we protect our shared tangible cultural heritage from biodeterioration and preserve it for future generations? The use of antimicrobial nanomaterials in the heritage science field represents challenging new research opportunities for chemists and materials scientists, where an integrated multidisciplinary approach is required to tackle the heterogeneous composition, uniqueness and value of each individual heritage piece.

INTRODUCTION

Artistic and architectural heritage in the form of books, paintings, clothing, historic monuments and buildings, among many other artifacts, represent significant tangible aspects of a community, region, or country's cultural heritage, from which current and future generations will embrace, study, and share. The preservation of our shared cultural heritage constitutes a global societal and economic priority,[1] yet the problems caused by lack of proper preservation of tangible heritage often only come to attention when tragedies occur, such as a fire, collapse, or water damage. However, there are many silent threats lurking permanently in our heritage items and buildings, far from the eyes of the great majority of people. Environmental aspects (humidity, temperature, light, CO_2 concentration, atmospheric pressure and pH) and geological conditions are two important factors affecting heritage objects; however chemical composition (organic *vs.* inorganic), the quality of the materials and ageing process, internal mechanical stress and biological colonization (originating specifically bacteria, fungi, algae, moss, lichen, and insects) constitute the other principle actors leading to the decay and deterioration of artifacts.[2]

In particular, biodeterioration - *"any undesirable change in the properties of a material caused by the vital activities of organisms"*[3] - poses a persistent problem in the conservation of cultural heritage. Contamination and spoilage of artefacts displayed in exhibition rooms or stored in depots is not exceptional; but rather frequent, in both old and newly built museums. Microorganisms such as bacteria, fungi, cyanobacteria and algae are highly proficient at inhabiting and decaying artistic and architectural heritage objects. Paper, leather, stone, textile, ceramic and glass are all blighted by this problem which incurs substantial difficulties for the conservation of cultural heritage.[2]

All types of historic artefacts in public museums and in private art collections are at risk of attack from fungi and bacteria: from textiles and leathers used for clothes and weaponry, to paper and books; not to mention architectural surfaces and stone monuments in outdoor environments along with mural paintings in churches, caves and catacombs.[4] In mural paintings, for example, microbial colonization can be the root cause a host of serious problems: discoloration of pigments and mortars, formation of stains and biofilms, salt efflorescence, exfoliation, cracking and disintegration of paint layers, formation of paint blisters, and degradation of binders that results in detachment of the paint layer(s). Fungi of the genera *Penicillium, Cladosporium, Alternaria, Curvularia, Dreschlera, Chaetomium, Fusarium, Trichoderma, Gliomastix, Aureobasidium*, are abundant in degraded mural paintings.[2,5] In fact, fungi are particularly threatening to heritage materials because their hyphae

1

undergo rapid proliferation and penetrate deep into all types of organic and inorganic materials. Meanwhile their spores, in a dormant state, are ever-present and available for germination when the local growth conditions become more favorable.[5] Furthermore, carboxylic acids produced through fungal metabolism (e.g., oxalic, citric, succinic, formic, malic, acetic, fumaric, glyoxylic, gluconic, and tartaric acids) can produce a highly aggressive second wave of corrosive chemical attack.

Consequently, the deterioration of artistic and architectural heritage results in immense financial damage for museums, local authorities and private collectors alike. More important still are the immeasurable cultural and societal losses caused by irreparable damage to unique artefacts, which represent our shared cultural heritage and identity, as well as an important social and economic resource for communities, regions, and countries. Although effective climate control, frequent cleaning and phenomenological monitoring all help to reduce biogenic damage to historical objects; there are other factors, such as acquired resistance to antibacterial agents and environmental damage (inadequate storage conditions, dampness, floods, transportation/relocation, etc.) that require a wider range of more suitable biocides.[6] Moreover, extreme proliferation of bacterial and fungal biofilms in closed spaces also present inherent (often severe) health risks for employees and visitors to museums and indoor public environments.[7] Unfortunately, the chemical community has not risen to this grand challenge and only a limited range of methods is available to disinfect recent and progressive microbiological deterioration to heritage objects. In addition, the available methodologies are frequently inadequate for cleaning and preventing long-term biofilm growth; plus certain coatings, paints and varnishes are themselves subject to microbial attack.[8]

Recently, there has been a drive for greater use of nanomaterials in the conservation of cultural heritage. Recent examples include their use for protecting stone monuments, textiles, murals, glass and paper. Of course, products based on nano-silver and nano-titania are studied frequently, but a variety of other readily available multifunctional nanomaterials have been shown to serve as alternative solutions to heritage biodeterioration issues.[9–11]

In this review, we provide a critical summary of the state-of-the-art in traditional and nanomaterials-based antimicrobial treatments for heritage items from a multidisciplinary perspective in order to include key considerations from the heritage conservation-restoration, materials science and microbiological standpoints. We aim to provide an overview and comprehensive analysis of the principal and most effective nanomaterial types and the most appropriate analytical biochemical techniques that can be used to accurately evaluate their antimicrobial properties, both *in vitro* in laboratory cell cultures under model conditions and *in situ* applied to samples in realistic settings. From a functional materials perspective, the first and most important step is to properly characterize the physicochemical properties of the nanomaterials (i.e. size, morphology, stability, and so on), however, the second stage requires a precise evaluation of the antimicrobial properties of the nanomaterials against relevant microorganisms to screen potential candidates, develop structure-property relationships and thus select the most active agent against to be used as a protective method in cultural heritage objects. Crucially, it is in the interests of the field to attempt to standardize analytical methods and demand fundamental materials characterization and more comprehensive antimicrobial assessment in order to have confidence in the data and to obtain comparable results. Choosing the wrong biochemical method or performing non-standard antimicrobial assays could lead to irreproducible and non-comparable results, or even to false negative or positive results. Consequently, at the heart of this review we outline standardized characterization techniques and biochemical assays/protocols used to evaluate antimicrobial compounds or materials more rigorously to help interested readers to select the most appropriate antimicrobial and antibiofilm methods, depending on the physicochemical properties and desired end use of each nanomaterial on different heritage substrates.

In most of the literature examples articles cited in this review, model substrate materials - most of which possess no true cultural or economic value - are used to evaluate the efficacy of antimicrobial treatments. In many cases these might include, for example, stone from the same quarries as those used in the construction of heritage buildings,[9,10,12] ceramic tiles or glass prepared using historically accurate production methods, filter paper, glass slides or freshly made bricks[13] or plaster, among others. These initial proof-of-concept model laboratory studies represent one of the first and most vital stages in evaluation, since any unforeseen and undesired effects that the antimicrobial treatments might cause to the heterogeneous character of an original heritage item preclude their direct use in mitigating biodeterioration. However, numerous antimicrobial nanomaterial treatments have also been evaluated on unique antique artefacts and architectures in their local environmental conditions at various cultural heritage sites around the world, which highlights the scope of the heritage biodeterioration problem as well as the global research effort being used to understand and address it (Figure 1). These reports include colorimetric measurements[14], *in situ* long-term monitoring of microbial recolonization[15,16] and studies of the biocidal efficiency of the treatments.[17,18] A complete description of the type of test performed on the cultural heritage items, their location, dating and the images references are provided in Table 1 in the supplementary information.

CURRENT TRENDS IN CHEMICAL PRODUCTS USED TO PREVENT BIODETERIORATION

Biodeterioration represents a serious on-going problem for the conservation of cultural heritage materials and consequently, a number of different preventive or indirect methods (such as environmental monitoring and control) and corrective or direct methods (mechanical, biological, physical or chemical) are currently used to reduce this threat. In this section, we focus on commonly used or commercial chemical biocides whose function is to inhibit or eliminate the growth of different microorganisms. Many of these products offer limited long-term effect, are highly toxic, and frequently are also corrosive. This is due to the fact that many of these biocides have not been designed specifically with the heritage material in mind, and

instead have been transferred from the agricultural or health sector. Moreover, some also present unwanted negative effects in the treated works, such as the discoloration of the heritage material or changes to its chemical and physical characteristics.[28]

Biocides can be both organic and inorganic where the latter provide a longer lasting action than organic products, which deteriorate rapidly, especially in outdoor environments. It is important to clarify that most of the biocidal products typically used in heritage conservation are on the whole quite ineffective in the elimination of microorganisms, and especially if they have been applied to materials exposed to outdoor environments. It is therefore necessary to regularly reapply products at regular intervals to prevent recurring recolonization of the material and also consider how acquired antimicrobial resistance can develop as microorganisms adapt to poorly chosen or ineffective biocides.[29]

When applying a biocidal product, it is important that it be chemically inert, stable, and colorless, in order to avoid interference with the material or its possible interaction with other compounds used in the intervention (such as consolidants). The cyto- and ecotoxicity must be also considered. Also, these materials must meet other requirements, such as resistance to external alteration agents, offer the potential for reversible application without causing damage to the substrate or in the case of stone materials, for example, maintain adequate permeability without obstructing their porosity.[30]

The effectiveness of the products used to prevent biodeterioration, as well as the results obtained with their application, will depend on a variety of factors such as the concentration, the stability of the product, the duration and the method of application, the type of solvent, the pH of the solution, the presence of cracks in the substrate or organic materials, the type of substrate and its content in water, the existence of wind and rain during and after the treatment, the ambient temperature, the light intensity or the colonization entity.[31]

It is also important to keep in mind that the chemical composition of some materials can modify the activity of these products, and may affect the longevity of action over time, depending on the material on which the biocide has been applied. These factors are often responsible for the success or failure of these products, in different interventions carried out worldwide. However, and even though commercial biocides are routinely used in the field of heritage conservation, it must be said that not enough studies have been carried out to assess their long-term efficacy, as well as the possible negative effects caused to the treated materials.[28]

The commercially available chemical products currently applied in the prevention of biodeterioration caused by heterotrophic and autotrophic microorganisms (such as lichen, algae, cyanobacteria, yeast, molds and bacteria), and plants (like moss and bryophyte), are summarised in Table 1.

Table 1. Commercial chemical products most commonly applied in the prevention of biodeterioration. Results tested under labora *"in situ"* **in real samples.**

Chemical classification	Active ingredient	Commercial name	Substrate & targeted microorganism	Efficacy	Examples/notes	Refs.
Alcohols	Ethanol		Stone materials, Paper	Contradictory results: not effective against *Trebouxia sp.*, *Gloeocapsa sp.* and *Chroococcus sp.* applied by brushing at 96%

Ethanolic solutions (30, 70, and 100%) revealed antifungal activity over short and long-term on paper | Granite (laboratory test)

Filter paper (laboratory test) | 32 |
			Bacteria, lichen, fungi, algae		Can act as a conidia activator	
Isothiazolones	2-methyl-4-isothiazolone- 3-one	ProClin ™ 950-Sigma Aldrich	Stone materials	Low efficacy: killed 20–40% algae cells	Laboratory test	33 34
			Algae			35

3

	2-octyl-2H-isothiazolon-3-one + didecyl dimethyl ammonium chloride in propanol + formic acid	Rocima™ 103- Dow Chemical	Masonry Wall paintings	Efficient applied by brushing, at 2% in water	Archaeological site at Ostia Antica, Rome (Italy)	14 17
			Fungi, algae		Must be properly diluted for use	
	5-chloro-2-methyl isothiazolinone, 2-methyl isothiazolone, methyl-benzimidazol-2-ylcarbamate + 2-n-octylisothiazolinone	Mergal™ S97-Troy Corporation	Stone materials	Effective	Laboratory test	
			Fungi, bacteria, yeast		Crystallization on stone pores which could make penetration of the solution difficult Good distribution on the surface	
	n-Octyl-isothiazolone		Stone materials	Effective, applied at 5% in ethanol	Agar diffusion tests, (laboratory test)	
			Fungi, algae, bacteria, actinobacteria			
	Di-n-decyl-dimethyl ammonium chloride, 2,N-ottil-2H-isotiazol-3-one + isopropanol + formic acid	Biotin™ T-CTS	Stone materials Ceramic materials Wall paintings	Contradictory results: not effective at 2% v/v Efficient, but recolonization occurred after 6 months	Palácio Nacional da Pena, Sintra (Portugal) Fishing House of the Marquis of Pombal Palace, Oeiras (Portugal)	
			Fungi, algae, lichen, bacteria		The use of anionic surfactants and hard waters should be avoided	
Mixes	Imidazole + isothiazolinone	Parmetol™ DF 12-Schülke & Mayr	Stone materials Plastic materials Textile Metals	High efficiency. Effective after 4 years	Angkor Wat, (Cambodia)	34 36
			Fungi, algae		Ethanol (70%) was applied first to destabilize. Avoid use with	

4

					alkaline solutions
	Iodopropynyl butyl carbamate + *n*-octyl-isothiazolone dissolved in 2(2´-oxydiethanol)	Biotin™ R-CTS	Stone materials Ceramic materials Wall paintings	Contradictory results: effective applied by brushing at 5% until saturation	Segovia cathedral cloister, (Spain)

Limestone, Classic Karst plateau (Italy) |
			Fungi, lichen, bryophyte, cyanobacteria, bacteria	Not effective after 4% v/v ethanol by brushing application	Barely miscible in water
	Sodium dimethyldithiocarbamate + sodium 2-mercaptobenzothiazole	Vancide™ 51-Vanderbilt	Stone materials Cotton Wood Paper Metals Plaster works	Good efficiency against bacteria and fungi, applied three times by brushing, at 1% in isopropanol	Marbles, (laboratory test)
			Fungi, algae, lichen	Good efficiency for 1-2 years	
	Tributyltin oxide + quaternary ammonium salt	Thaltox™ Q-Wykamol Ltd.	Stone materials Woods lacking artistic interest Wall paintings	Effective applied by spraying, but after three years almost completely washed out by the rain	Copan monuments, (Honduras)
			Algae, moss, lichen		Reapplication every four to eight years
Oxidizing agents	Calcium hypochlorite		Stone materials	Good efficiency applied at 5% in hot water	Palace of Saints George and Michael, Corfù, (Greece) [37] [37] [34]

Washington Legislative Building (USA) |
| | | | Algae, cyanobacteria, lichen | | Its activity is not maintained long (recolonization after 1 year) |
| | Hydrogen peroxide | | Stone materials | Contradictory results: not effective applied by brushing, at 10% in water | Historic cemetery of Drapano, Kefalonia, (Greece)

Slovenian caves |
| | | | Algae, bacteria, lichen | Effective applied by sprayed at 15% (v/v) in water and carbonate/ | Can bleach materials |

					bicarbonate buffer solution	
	Sodium hypochlorite		Stone materials Water saturated wood	Good efficiency applied at 3,5% in hot water	Palace of Saints George and Michael, Corfù, (Greece)	
			Algae, cyanobacteria, lichen		Interferes with some stone materials, (excessive bleaching or secondary yellowing action) May react with wood lignin Its activity is not maintained long	
Phenolic compounds	Diuron 3-[3,4-dichlorophenyl]-1,1-dimethylurea	Preventol™ A6-Lansess	Plasters	Effective	Laboratory test	35 38
			Algae		It can produce a darkening of limestone surfaces and a less detectable change on granite surfaces	
	n-butyl-1,2 benzoisotiazolin-3-one	Algophase™-Phase	Stone materials	Good efficiency at 3% v/v in water	Acropolis, Athens (Greece)	
			Bacteria, algae, lichen, fungi		A first application is recommended to devitalize microflora, (3-5% benzalkonium chloride in aqueous solution), followed by the mechanical removal of necrotized residues	
	Parabens (p-hydroxybenzoates) + calcium propionate		Paper	Very effective for inhibition using methyl paraben at 0.5% or propyl paraben at 1% with 5% calcium propionate	Laboratory test	
			Fungi		It can be used to avoid fungal growth and as a treatment when is already infected	

	Resin alkyleneoxide+ alkylaminotriazine+ N-(3,4-dichlorophenyl)-N,N-dimethyl urea + denatured alkyl trihydroxyben-zene polyoxide	Koretrel™-Tokai Concrete Industries Co., Ltd.	Stone materials	Highly effective	Trieste Karst, (Italy)	
			Lichen		It produces color changes in the surfaces and the absorption of water by the capillary action of certain types of stones	
Quaternary ammonium compounds	Alkyl dimethyl benzyl ammonium chloride	Preventol™ R50, Preventol™ RI80, Preventol™ R90-Lannxess, Hyamine™ 3.500-Lonza Company, Céquartyl™-Rhone Poulenc	Stone materials Wood Ceramic materials	Contradictory results: not effective at 4% w/v in ethanol	Acropolis, Athens (Greece)	38 17 39 14
					Fishing House of the Marquis of Pombal Palace, Oeiras (Portugal)	
			Bacteria, lichen, fungi, algae	Efficient, but recolonization occurred after 6 months	It is recommended to apply another coat (after 24 hours)	
					Corrosive to metals	
	Benzalkonium chloride	Wet & Forget™-Wet & Forget Ltd.	Stone materials Wood	Contradictory results: not effective at 1% Effective at 10% in water	Statues of La Recoleta Cemetery, Buenos Aires, (Argentina)	
					Feilai Feng limestone, (China), (laboratory test)	
			Bacteria, lichen, fungi, algae		Corrosive and toxic	
	Benzalkonium chloride + 2-phenyl-phenol	Acticide™ 50X-Thor	Stone materials Wood	Not effective at 5%.	Feilai Feng limestone, (China), (laboratory test)	
			Bacteria, lichen, fungi, algae			
	Didecyl dimethyl ammonium chloride + excipients		Wood Bricks Plaster	Contradictory results: very effective against bacteria and low efficacy against molds, after 28 days	Wood and brick fragments from Auschwitz II – Birkenau State Museum, (Oświęcim, Poland), (laboratory test)	
			Bacteria, molds	Effective after 12 months	Laboratory test	

	N, N-dodecyl-N, N-dimethylammonium	New Des™ 50-CTS	Stone materials Ceramic	Contradictory results: effective. Higher efficacy against Gram-positive bacteria Low efficacy applied at 2% and not effective for killing phototrophic organisms	Laboratory test Processional cloister of the Monastery of San Martiño Pinario (Spain)	
			Bacteria, biofilm, algae		Could be used, in a lower concentration range than that indicated by producer Decrease tension superficial and interfacial of the water in which it has been dissolved Caused visible color changes on a granite wall	
	N,n-didecyl-n-methyl-poly(oxyethyl) ammo-nium propionate + alkyl-propylene-diamine guanidium acetate	Anios DDSH™-Anios Laboratories	Compatible with all kinds of materials	Not efficient	Walls on buildings in Sintra, (Portugal)	
			Yeast, lichen, algae, moss, bacteria		Ready-to-use spray foam	
Essential oils	Thyme oil	Doterra Thyme oil	Bacteria, fungi	Reduction 12-100% depending on the microorganism	Paper (books)	18

Essential oils, although not directly marketed as antimicrobials for heritage conservation, are worthy of special mention since they represent relevant antibacterial agents used routinely in other areas, e.g. active ingredients in food packaging, and are likely to receive added attention in the coming years. The volatile compounds present in essential oils extracted from plants present good antimicrobial activity against target microorganisms and are eco-friendly, naturally occurring biocides. However, their high anti-oxidant capacity and migration issues should be considered. For a more comprehensive analysis on the topic, readers are referred to the following reviews.[40,41] Pioneering reports on nano-encapsulated essential oils in heritage conservation are discussed at the end of the following section.

ANTIMICROBIAL AGENTS IN CULTURAL HERITAGE CONSERVATION

Antimicrobial nanoparticles (NPs)

The antimicrobial properties of materials such as silver, zinc oxide or copper have been known since time immemorial. For example, it is known that the water was already stored by the Egyptians in copper or silver containers to make it drinkable, or that during the exploration of the Wild West, silver coins were used to preserve the freshness of milk and to conserve drinking water against algae and bacteria. In the case of ZnO, it is also known that it was used as far back as 2000 BC, as a treatment for boils and injuries. The antimicrobial properties of such materials increase upon reductions in particle size down to the nanoscale, because of fundamental changes in the physics of electron properties in the crystalline solid, combined with the better interaction with cells and intercellular components such as proteins, DNA, ion channels, etc.

Nanomaterials have been thoroughly investigated as antimicrobials in applications as diverse as water disinfection, food packaging, and healthcare. It is perhaps then not surprising that during the last decade, nanomaterials have also emerged as antimicrobial coatings and treatments in the field of heritage conservation. However, this area is still in its infancy and only a limited number of reports have been published over the last 10 years. As part of this review a total of 84 papers have been

identified, primarily using the keywords <nanoparticles>, <antibacterial> <antifungal> <antimicrobial> <biodeterioration> <biocidal> <bactericidal> <fungicidal> <cultural heritage> <relics> and <monuments>, which equates to 15, 27 and 42 papers in the periods 2010-2013, 2014-2016 and 2017-2020, respectively. Nanomaterials have also been broadly used in heritage science for deacidification, consolidation and cleaning processes,[42] where a number of commercial products already exist, however such studies are outwith the scope of the present review.

In the first instance, in order to be considered as a potential candidate for application in the heritage conservation field, a nanomaterial should present a potent and long-lasting antimicrobial activity, and also display low cytotoxicity and ecotoxicity.[43] This presents a challenge for the development of novel biocides based on nanomaterials, since the long-term toxicity or the effect on the environment of many of them are still under study. Moreover, the Guidelines of Conservation state that, following a coating application, the aesthetic appearance of the heritage object (color, texture) should not be perceived to have been changed and any intervention must be susceptible to be reverted in the future,[44] which also implies a challenge in the design of nanomaterials-based coatings. Finally, regarding the commercialization of conservation products based on nanomaterials, the synthesis processes must be as simple and reproducible as possible, and the price of raw materials should be low, in order to develop competitive market-ready nanomaterial-based formulations.

Nanoparticles of titanium dioxide (TiO_2), silver (Ag) and Zinc Oxide (ZnO) are, in this order, the most recurrently applied nanomaterials to prevent or treat microbial colonization of heritage substrate materials. Other frequently reported nanoparticles include silicon dioxide (SiO_2), copper (Cu), magnesium oxide (MgO), zinc-derivates such as calcium zinc hydroxide dihydrate, $Ca[Zn(OH)_3]_2 \cdot 2H_2O$, carbon nanomaterials (graphene, graphene oxide and fullerene) and layered double hydroxides (LDH). To date, nanomaterials have been applied over a range of cultural heritage items and architectures, either model replicas or original artefacts, which include those originating from animals (parchment, wool and silk tissues) and plants (paper, wood, cotton fabrics) plus those obtained from inorganic resources (stone, glass, plaster). Figure 2 provides an overview of the occurrence of publications that have used antimicrobial nanomaterials to prevent biodeterioration on different types of heritage materials. Our analysis shows that the most studied type of nanomaterial has been nano-TiO_2, which has been applied to most of the heritage substrates, but is followed closely by Ag NPs. On the other hand, stone - in its many different varieties - has undoubtedly been the most studied heritage substrate material, being the focus of a total of 47 relevant publications to date.

Synthesis and properties of the nanomaterials tested as biocides in heritage

Wet chemistry methods (sol-gel, soft-chemistry and autoclave processes) have been utilized to synthesize up to 51% of nanomaterials reported herewithin (see Figure 3).[21,45] Some of these syntheses have been complemented with a calcination treatment (9%), to produce oxides such as TiO_2, MgO or ZnO.[10,46] Commercial materials have also been broadly utilized (in up to 27% of the published literature), but generally have been limited to TiO_2, ZnO and Ag NPs.[47,48] The availability of these three materials for purchase directly from commercial suppliers has undoubtedly rendered them as some of the most studied antimicrobial materials in the heritage conservation field, as previously shown in Figure 2. Although a variety of other synthetic approaches have also been used, including the use of microorganisms, so-called biosynthesis (8%), used for ZnO,[49] Cu NPs[50] and Ag NPs[16,51]. Electrochemical methods (4%) have also been used to prepare ZnO[19] and Cu NPs[52] and sonochemical methods (4%) are currently trending as a convenient, rapid and cost-effective route to obtain nanoparticles such as ZnO.[53,54] Physical vapor deposition has also been used for the synthesis of In-doped ZnO.[55] Finally, it is worth mentioning that 6% of the reviewed publications failed to even specify the origin or synthetic procedure for the nanoparticles used in the study.

In these studies, the heterogeneous nature of the synthesis methods (including the commercial materials, whose processes remain undisclosed) causes a broad distribution of the average particle size, ranging from diameters of a few nm to more than 100 nm. Moreover, the difference of concentration applied over the specimen, ranging several orders of magnitude, and other factors (size distribution within each sample, particle aggregation, type of substrate, environmental conditions, type of microorganisms) involve so many variables that it is not possible a direct comparison or a statistical analysis of the results in terms of the effectiveness or durability of the conservation treatment. In order to be able to compare the effectiveness of the treatments, the heritage conservation research field would benefit greatly from establishing a series of standardized protocols or assays to evaluate the antimicrobial activity of nanoparticle coatings or treatments. Such considerations are discussed in more detail in the final section of this review.

Nanomaterial application methods

If choosing the right antimicrobial nanomaterial is essential, the selection of the most appropriate mode of application (considering the properties of both the nanomaterial and the heritage material surface) is the next key step in the process. The techniques most commonly used so far have been brushing[56,57] and spraying[13], which are adequate for most substrates and ensure a homogeneous nanoparticles coating, as well as other like immersion,[58] drop-casting,[59,60] spreading with a spatula,[61] misting,[23] capillary absorption[62] and even spin-coating for a glass test specimen[63]. In most of the studies, the nanomaterials were dispersed in polar protic solvents which quickly evaporate, like water or ethanol,[12,64,65] however some studies took further the idea of stabilizing the coatings versus environmental conditions and organic or inorganic matrices were incorporated for this purpose. These includes commercial varnishes[46] and consolidants[15,19,22,50,52,56,60,63,66–68] and natural and synthetic polymers such as polyvinyl butyral,[47] starch,[54] siloxanes,[59,62,69,70] acrylic resins,[27,71] wax,[72] estearates,[73] nanocellulose,[53]

9

hydroxyethylcellulose[74] and hydrogels[48,75]. These hybrids generally enhanced the attachment properties of the nanoparticles, thus improving the longevity of the antimicrobial coatings.

Types of microorganisms used to evaluate the potential of antimicrobial nanomaterials

Following the nanomaterials deposition on the substrate, the antimicrobial efficiency must be assessed. Biodeterioration processes are usually caused by a wide and heterogeneous community of colonizing microbes constituted by prokaryote (bacteria and cyanobacteria) and eukaryote microorganisms (fungi and algae), where the presence and prevalence of one type of microorganism over another will typically depend on the available nutrients and on the environmental conditions to which they are exposed. For example, the availability of natural light naturally favours the proliferation of photosynthetic microorganisms such as algae and cyanobacteria; while in dark and humid conditions heterotrophic microorganisms like molds and bacteria will be the more prevailing and abundant species.[16,18,21,76] The microbial colonization of different types of heritage object, from stone monuments and other architectures to paper heritage, textiles, metals, and other works of art, is discussed in detail in several excellent books and reviews.[2,77,78] Briefly, to better understand this process, here we will take an outdoor stone surface as an example (Figure 4), where the pioneering colonizers are typically autotrophic cyanobacteria and algae that can obtain their nutrients from the inorganic compounds on the stone surface and the sunlight. Once the autotrophic microorganisms have obtained a foothold on the stone surface, the heterotrophic microorganisms can establish themselves as secondary colonizers, obtaining organic nutrients from the autotrophic cyanobacteria and algae.[78] We recommend that interested readers consult the aforementioned texts for detailed discussion of the different types of microorganisms and the environmental conditions favouring their proliferation.

To date, a variety of different microorganisms relevant to the decay of heritage objects and architectures have been used to evaluate the efficacy and potential of antimicrobial nanomaterials.[79] Figure 5 shows the occurrence of bacteria, fungi and algae in the studies in this review, specifying the type of heritage substrate and the type of nanomaterial. Fungi (in particular molds) and bacteria are the most studied microorganisms. Regarding fungal studies, *Aspergillus niger* is by far the most common of the more than 30 fungal species studied, which also includes *Penicillium oxalicum*, *Candida albicans* and *Alternaria alternata*, among others. The biocidal activity of nanomaterials has been tested against more than 20 types of bacteria, with *Escherichia coli* being the most common strain, together with *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus cereus*. It is important to remark that, even though bacteria are not always the main colonizing microorganisms, they present some characteristics (e.g. rapid growth, can be cultured and evaluated in the laboratory through reproducible protocols) that make them highly suitable as model microorganisms for the first *in vitro* assays. Besides, some very recent studies have demonstrated the presence of different bacterial species in heritage objects, deposited through vectors, such as insects, or even due to human contamination during restoration or other processes. This research opens the door to the study of a new source of contamination, making non-environmental bacteria relevant to biodeterioration processes.[80] It is worth mentioning cyanobacteria as a special case of study. While this bacteria domain represents a relevant pioneering colonizer of outdoor heritage objects, very few studies were carried out with this microorganism. Just six papers of the consulted bibliography studied the effect of Ag NPs[13,61,81] and TiO$_2$ NPs[14,21,82] on cyanobacteria. *Chlorella vulgaris* is the most utilized algae from a list of more than ten different algal species. Only a few studies concerning treatments specifically for lichens have been performed -not included in the figure- over stone with TiO$_2$[14] and Cu NPs[15]. Algae, lichens, cyanobacteria and moss require of a supply of water and light to grow, and so the research projects involving these microorganisms were focused in stone and building materials in outdoor environments. Yet, studies preventing algal growth on wood, glass, and metal in cultural heritage objects are non-existent, despite the fact that algae are known to be pioneering microorganisms in extreme environments.

Despite the fact that most studies employ easily culturable laboratory strains, some have gone as far as to analyze the type of microorganisms present in the heritage object, which were identified by traditional culture methods (microscopy, gram staining, enzymes activity) and molecular techniques (PCR amplification, sequencing).[15–18,20,21,26,27,61,76,82,83] In these cases, the effectiveness of the biocidal treatments was tested either *in vitro* (on culturable strains) or *in situ* (on the heritage item itself), which provide the added value of dealing with the microorganisms that cause the biodeterioration of the heritage material. Some studies were carried out under the exact environmental conditions that caused the deterioration[17,20] while others have used similar conditions simulated in the laboratory.[21,82]

The type of studies that have been performed are based primarily on the identification of the microorganisms present in real samples by sequencing the 16S rRNA gene, and the determination of the minimum inhibitory concentration (MIC) of the compounds against the different microorganisms. Our analysis has shown that Broth Dilution Methods and Agar Diffusion Test were the preferred means of determining the MIC, and, as we will explain later in the review, these may not be the most suitable methods depending on the characteristics of the compound. Although some studies performed more accurate microbiological assays to determine the activity of the compounds[76] there is clear a need to establish more standardized protocols to determine the antimicrobial and antibiofilm properties of a compound or nanomaterial *in vitro*, but also over the heritage material being protected. To this end, this weakness is addressed in section 4 and a comprehensive description of appropriate techniques is detailed.

Nanomaterial and nanomaterial-heritage substrate characterization techniques

10

This section overviews the principal analytical techniques that have been used to characterize the nanomaterials, as well as their interaction with the substrate heritage material.

Size and morphology of the nanoparticles are most often reported using images from scanning electron microscopy (SEM) and transmission electron microscopy (TEM).[26,56] Nanoparticle size distribution in solution has been frequently analyzed by dynamic light scattering.[61,81] The reliability of this technique, however, is limited to particles with spherical shape. This technique is affected by aggregation and, therefore, can be used to study the stability of the nanoparticles dispersion prior application.[21,65] It must be highlighted that, despite the fact that nanoparticle antimicrobial activity is often size-dependent,[84] *ca.* 30% of the publications cited herein contain no such analysis of the nanoparticle dimensions.

Crystallinity of the nanoparticles, defects, and doping: TEM and X-ray diffraction (XRD) provide key information about the crystallinity of the samples, the presence of defects and doping.[56,85]

Compositional analysis: additional techniques present in most electron microscopes, such as energy-dispersive X-ray spectroscopy (EDX) and electron energy loss spectroscopy (EELS), provide insights into the elemental composition of the nanoparticles and substrate.[26,50] For quantitative compositional analysis, the preferred techniques are X-ray photoelectron spectroscopy (XPS), elemental analysis, or Inductively Coupled Plasma (ICP).

Homogeneity and degree of penetration of the nanoparticles coatings in the substrates have been extensively studied by SEM, EDX[60,71] and also by XPS.[63]

Presence and growth of microorganisms on different surfaces hava been characterized by SEM and optical microscopies. For samples with high sensitivity to vacuum or those which cannot be coated with a conductive layer, environmental scanning electron microscopy (ESEM) is the preferred tool.[11,12] This technique operates under a controlled gaseous atmosphere instead of vacuum and allows the imaging of wet samples where the microorganisms are under conditions similar to their natural state. Optical microscopies are non-invasive techniques, useful to study the structure of the substrate, the microorganisms and the antimicrobial performance of the coatings.[26,48] Combined with cells staining (for example, death/alive bacteria) are a useful tool to evaluate the effectiveness of a given treatment. Digital image analysis is a useful tool to evaluate the percentage of the surface of a heritage item that has been colonized by the microorganisms that cause a color variation on the same, such as algae or fungi.[81,82] Spectroscopies using infrared, ultraviolet-visible radiation and photoluminescence have also been reported as convenient and universal methods for the characterization of the nanoparticles and coatings.[22,56,86] Fourier transformed infrared (FTIR) spectroscopy has been revealed as a useful tool to study the evolution of organic substrates during accelerated ageing studies.[22,47,53]

Color variation: As mentioned previously, any mitigating biodeterioration prevention coating must not alter the aesthetic appearance of the heritage object to a noticeable degree. Most of the articles in this review address this issue by performing colorimetric studies. The surface color is characterized before and after the nanomaterial application by spectrophotometric techniques, and the chromatic change calculated as ΔE in the CIE 1976 L*a*b* color space.[10,12,45,52,60,68,74] Most authors refer to a $\Delta E \leq 1$ as being a perceivable change, but assume $\Delta E \leq 5$ to be acceptable.

Ageing studies: Ideally, an antimicrobial coating should possess a long-lasting effect However, most research projects are time-limited, and only in some cases it has been possible to study the performance of the treatment *in situ* during a period of a several years. Some examples found in literature include TiO_2 on glazed tiles (two years),[17] TiO_2/wax on marble in underwater marine environment (two years)[72] and Cu NPs mixed with commercial consolidants on several inorganic substrates (three to eight years).[15,68] Nevertheless, in most of the projects it is necessary to perform accelerated ageing tests to assess the durability and resistance of the antimicrobial coatings within the project time frame, as follows:

- **Biofouling accelerated growth:** tests have been performed by introducing the coated samples within climatic chambers with controlled conditions of humidity, temperature and simulated sun light. Water supply was ensured by using sprinkling systems,[82,87] or direct immersion.[21,61] More recently, Becerra *et al.* have proposed capillarity system for wetting the substrate. This is considered a more realistic simulation since multiple applications of culture are avoided compared to sprinkling systems.[81]

- **Accelerated environmental factors (light, temperature & humidity)**: the use of climate chamber studies, or similar, is also common.[53] The exposition of the substrates to UV light to simulate solar aging has showed that TiO_2/Mg(OH)$_2$ hybrids improve the tensile strength resistance of paper under UV radiation,[56] or that the presence of Ag NPs do not alter the stability or the color of cotton fabrics during accelerated light ageing.[88] The avoidance of color fading in pigments/colorants and the improvement of tensile strength in paper by ZnO and TiO_2 nanoparticles[22,46,66] has also been studied. Other authors reported a positive effect on the stability of the organic matrix that contains the nanoparticles.[27,62,67] The photoactivity of ZnO[12,62] and TiO_2[67,86,87] was also evaluated by the discoloration of rhodamine or methylene blue solutions upon UV-vis light irradiation.

- **Freeze-Thaw cycles:** Cycles of freezing and thawing[89] and heating treatments (60-90 ºC) inside chambers,[22,62] in occasions with high, controlled humidity[47] showed improved performance of the nanoparticles-coated substrates versus the uncoated ones. Other ageing studies include the evaluation of the treated surface after several months exposed to environmental conditions to check the decrease of the accumulation of dirtiness on TiO_2-treated substrates.[46]

- **Nanoparticle-release studies:** It must be highlighted here that the evolution of the coatings regarding the release of nanoparticles, lixiviates of the organic matrices, and the possible toxicity related to this has only been studied in the release of Zn from ZnO *via* a rainwater mimicking study.[63] In fact, the evolution of the treatments is a critically important issue to sustainable conservation practices because of the implication on the health of curators, museum

11

visitors, and the environment.[90] It is our view that through toxicological and ecotoxicological research should be developed in parallel to nanomaterial research in heritage conservation to ensure their safe and sustainable use.

Heritage substrate material studies: Broadly speaking, the properties of the heritage materials (substrates) are unevenly characterized in the literature cited in this review. Stone is usually analyzed by a comprehensive set of techniques, which include several of the following: porosimetry, surface area, roughness, water vapor permeability, capillary water absorption and static contact angle,[12,15,45,50,58,62,67,68] while paper usually includes basic tensile strength measurements to evaluate changes in resistance.[46,66,74,83] However, it must be stated here that is should be necessary to perform a more systematic study of the heritage object in terms of composition and structural arrangement. In general, for all types of materials, certain properties must be present in any study, such as their typology, size, composition, pH, density, as well as their color. But in addition to this, there are other characteristics specific to certain materials, simple to determine and that provide great information, as for example the grammage of the paper.[91]

Looking to the future, it is becoming increasingly clear that studies should also involve the use of large scientific facilities, like Neutrons[92] & Synchrotron radiation[93,94], whose use in heritage science has increased considerably in recent years. Valuable information about the porosity and morphology of samples can be provided by computer tomography-based techniques.[95] The use of such facilities enables non-destructive analysis with high spatial resolution and accurate compositional information, as well as high resolution imaging of the coatings and substrates, therefore providing a better characterization and deeper understanding of surface properties and enhanced information on ageing studies. It is also important to note that the Heritage Science community is supported by the European Research Infrastructure for Heritage Science (ERIHS), which supports research on heritage interpretation, preservation, documentation and management through transnational access (TNA) to a wide range of high-level scientific instruments, methodologies, data and tools for advancing knowledge and innovation in the field of Heritage Science.

TiO_2 NPs

TiO_2 is the most utilized nanomaterial in heritage conservation, as it is inexpensive, chemically stable and environmentally benign. The well-documented antimicrobial properties of TiO_2 rely on its photoactivity: this semiconducting material releases reactive oxygen species (ROS) upon exposure to UV light or solar radiation. ROS are formed from molecules present in the atmosphere (O_2 and H_2O) and include superoxide anion radicals ($\cdot O_2$), hydroxide radicals ($\cdot OH$) and hydrogen peroxide (H_2O_2), which are capable of killing microorganisms by oxidative attack of their cell membrane.[96]

Fonseca *et al.* were the first to report the use of nanoparticulated TiO_2 to prevent biodeterioration on heritage items in 2010, demonstrating the efficiency of pure and Fe^{3+}-doped 20-nm anatase to decrease the growth of cyanobacteria and algae on mortars.[14]

The majority of reports in the literature show that TiO_2 is bacteriostatic and generally only slows down the growth of microorganisms on the surface of heritage items when activated by UV or visible light, and it is typically not strong enough for a complete biocidal action.[20,62,66,67,97] Further, TiO_2 presents null[58,61] or poor[26,47] antimicrobial activity in the absence of light.

TiO_2 has been shown to diminish the growth of algae on bricks and stone in under water environments and when subject to humid conditions (Figure 6, a, b).[82,87] Similarly, Coutinho *et al.* reported the partial detachment of the biofilm in the glazed wall tiles of Palacio da Pena (Portugal) under natural sunlight conditions even two years after application.[17] Afsharpour *et al.* designed a TiO_2-coated glass box for paper art works preservation[66] which protects the objects inside of damaging effects of microorganisms, UV light and pollutants (Figure 6c).

An interesting approach was developed by De Filpo *et al.* (Figure 6a) whereby a removable coating of TiO_2-loaded gellan gum was applied over parchment producing a dual antimicrobial functionality: first, organic contaminants (spores, hyphae) were removed after being trapped in the 3D polymeric network and, second, UV light-activated TiO_2 nanoparticles had a biocidal effect and no fungal regrowth was observed, although the testing time for the fungal growth was limited to 15 days. One particular advantage for this treatment is its reversibility, since the gellan gum can be removed after finishing, leaving no residual gel on the parchment surface. Although, some of the TiO_2 nanoparticles were observed to remain in the parchment which continued to function as antimicrobial reservoirs afterwards.[48]

One of the limitations on the use of TiO_2 as antimicrobial is that, due to its bandgap, this material absorbs photons mainly in the UV region. La Russa and Ruffolo pioneered the use of Ag-doped TiO_2 to prevent heritage deterioration, where the presence of Ag shifts the absorbance of photons into the visible region and enhances its photoactivity under sunlight.[85,86] Nanoparticles of TiO_2 and Ag-doped TiO_2 were synthesized by wet-chemistry methods and their capacity to prevent the growth of microorganisms on marble slabs under a simulated marine habitat was studied. No microbial colonization was observed after 72 h in samples containing Ag-TiO_2, in contrast to untreated samples, which were extensively colonized by algae. Regarding samples treated with only TiO_2, these authors reported a remarkable higher activity for TiO_2 against bacteria at a concentration of 0.01% vs 0.1% (w/w in distilled water), reporting a 0% bacterial survival at 0.01% versus a >19% survival for the higher TiO_2 concentration.[85] Other authors have studied doping TiO_2 with Fe, Sr,[86] and Ce[70], which resulted in complete inhibition of bacterial growth on stone for Sr-Ag-TiO_2 and Ce-TiO_2. In this instance it must be highlighted that, although doping TiO_2

12

improves the performance of the coatings, the absorbance of photons in the visible region causes color variations which might alter the color of the heritage item.

Another strategy to improve the antimicrobial properties of TiO_2 has been to explore the synergic effects arising from using a combination of nanomaterials. Hybrids of $Ca(OH)_2/TiO_2$,[58] ZnO/TiO_2,[47] and Zn:Al LDH/TiO_2 [98] were employed as effective antifungal agents on limestone, wood and bricks, respectively. ZnO/TiO_2 showed an effective protection of wood even during ageing tests performed at high relative humidity and using further UV irradiation.[47] For their part, Vidakovic *et al.* reported that the ageing of the TiO_2/LDH coating is substrate-dependent and the treatment should be renewed every 4-7 months.[98]

Hybrids of TiO_2 and Ag NPs showed superior antimicrobial activity, achieving a complete or significant reduction (>98%) of microbial growth,[21,61,67,69,72,82,85] although authors state that the inherent antimicrobial activity of Ag NPs might be key in these cases. The antimicrobial properties of Ag NPs by themselves will be discussed in the following section.

Interesting enough, several authors have observed that the testing conditions and the properties of the substrate strongly determine the efficacy of the antimicrobial treatments. As stated by Graziani *et al. "the efficiency of any coating can-not be assessed without testing them directly on each substratum they could be applied."* [13] Ruffolo *et al.* showed that, for a given TiO_2 coating on plaster in a heritage site, the presence of soil humidity decreased the antimicrobial effectiveness of the TiO_2 when applied close to the ground in comparison to upper zones.[20] Porosity and roughness of the heritage substrate are also a key factor in treatment effectiveness. Graziani *et al.* studied the effect of surface roughness on a TiO_2 treatment against algae on fired bricks with 36% porosity. The initial surface roughness of 8 µm was smoothed to 1 µm with sandpaper. Even in absence of a treatment, a 20% lower algal coverage was observed in the clay bricks with lower roughness. The treatment of the bricks with 4-nm diameter TiO_2 showed no decrease in the algal coverage in the rougher sample, however, a reduction of algal coverage of 40% was observed in the lower roughness bricks compared to the blank.[87] The same authors observed a similar effect regarding porosity, where the initial algal coverage were 95% and 20% for untreated stone specimens with a porosity of 37 and 19%, respectively. Antimicrobial treatments in the stone with 19% porosity showed an algal coverage of 3.6% for TiO_2, while it reached 6.7% with Ag NPs/TiO_2, and 8.2% with Cu NPs/TiO_2. However, no antibiofouling effect was observed for these treatments on the 37% porous stone.[13] Other authors reported a similar effect of the porosity/roughness on ceramics and limestone.[58] The porosity of the substrate contributes to retain nutrients and moisture while decreases the coating efficiency due to diffusion of the nanomaterials within the pores. Roughness is also important since some microorganisms, like algae, require of asperities to adhere. Moreover, Becerra *et al.* noted that the chromatic changes for a given treatment are related to the porosity, as the diffusion of the nanoparticles into the pores led to a smaller ΔE in more porous materials.[61]

All these examples illustrate the difficulty in developing an antimicrobial coating on a proof-of-concept sample in the laboratory, before even taking into consideration its successful implementation on a real heritage surface in more realistic natural setting, outside of the laboratory.

Ag NPs

Ag NPs present broad-spectrum antimicrobial activity resulting from the attack on multiple microbial cellular processes: Ag NPs increase the oxidative stress *via* ROS formation, interfere with nutrient transport processes in the cytoplasmatic membranes and disrupts metabolic processes. This activity is boosted by the release of Ag^+ ions, which inhibit DNA replication and inhibit enzymes and peptides, which eventually leads to the microorganism death.[99]

Gutarowska *et al.* pioneered the use of biocidal Ag NPs in heritage preservation, demonstrating their potential as a powerful disinfectant for the surface of archival documents and historical objects.[76] These authors isolated microbial strains from the air and surfaces from different museums and archives from Warsaw and Lodz, Poland, (see Table 1 in suppl. Info) and removed up to 94% of those microorganisms using a 45 ppm loading of Ag NPs. Thereafter, the Lodz team optimized a method to disinfect heritage objects within a misting chamber (see Figure 7a) in which Ag NPs from a dispersion were nebulized over textiles, paper or canvas.[100] These authors found that this misting-disinfection process was sensitive to the degree of relative humidity, since moisture eases the penetration of the nanoparticles in the microbial walls. Moreover, these authors also observed that vegetative cells (bacteria and mycelium) are more sensitive to the antimicrobial effect of Ag NPs than the corresponding bacterial or fungal spores. Further experiments with pre-Columbian fabrics from Peru (wool, cotton and sisal) showed that the reduction of microbial number depended on the type and initial amount of microbial species: while it ranges 30.8–99.9% for some species of bacteria and fungi, some other species (mostly endospore-forming bacillus) were insensitive to Ag NPs-misting treatments.[23]

Reports by other authors have shown that Ag NPs effectively reduce the cell viability of bacteria on sandstone by 80%, [59] biofouling on mortar by 40% under humid conditions (see Figure 7b),[73] and the growth of aerial algae on facades by 98%.[101]

The amount of Ag NPs that can be used for an antimicrobial treatment is notably limited by the color change produced by the application of particles that absorb strongly in the range of 390-470 nm. Becerra *et al.* reported a limit on the efficiency of the antimicrobial properties of Ag NPs that can be achieved in low porosity stone due to esthetical considerations.[61] Essa *et at.* reported a complete growth inhibition of microorganisms *in vitro* using a 60 µg/mL suspension of Ag NPs, although to avoid

color change on stone surfaces the maximum concentration that could be used as part of *in situ* assays was 40 µg/mL (Figure 7c). To mitigate this undesired color change, Ag NPs were further combined with a silicon polymer, which sharply reduced the growth of both bacteria and fungi on the coated stones (*Streptomyces parvulus* growth displayed a reduction of 98.6% and the growth of *A. niger*, determined visually, was almost inexistent).[71] The color limitation associated with Ag NPs can be moderated by embedding them in a removable hydrogel,[102] similarly as described for TiO$_2$ in the previous section.[48] In this case, Ag and AgCl NPs were embedded within a poly(vinyl)alcohol-borax hydrogel and authors demonstrated an effective elimination of the microorganisms present in the stone. However, the microorganisms were not isolated for identification, and so the absence of quantitative antimicrobial data means that the results should be treated with caution.[102]

Carrillo *et al.* carried out an *in vitro* study on the effect of the size and concentration of biosynthesized Ag NPs against fungal and bacterial strains isolated from the citadel of Teotihuacan (Mexico). In this particular case, nanoparticles of 12 different sizes in the range of 39-367 nm were tested, but no clear trend was observed for the inhibitory effect *vs.* the nanoparticle size. However, these authors found that the effectiveness was dose-sensitive, reaching a maximum of effectiveness above 90% against some bacteria, although a clear dose-effect trend against molds was not observed. In a further *in vivo* study, the reduction of microbial colonization ranged from 90 to 98% for *Pectobacterium carotovorum* and *A. alternata* on three different types of heritage building materials (stucco, basalt and calcite).[16] Gámez-Espinosa *et al.* reported the 100% inhibition on the growth of *Aspergillus versicolor* and *Cladosporium cladosporioides* on bricks coated with a 2% Ag NPs loading on silane.[51]

Pietrzak *et al.* used ancient books from a Public Library in Lodz (Poland) and the National Archive in Prague (Czech Republic) to compare the efficiency of three different antimicrobial treatments: Ag NPs, essential Thyme oil and a low temperature plasma treatment (generation of ROS from air by electric discharges). The Ag NPs were found to be the most effective against bacteria, which underwent a 60-100% growth reduction *vs.* 12-100% for the other treatments, although all three approaches showed a similar performance against fungal contaminants (0-98.8%). For all three treatments, the effectiveness was lower for fungi than for bacteria and depended on the type book, the area of sampling and the tested microorganisms. Efficiencies as low as 0% and as high as 100% in viability reduction tests were reported for the three types of treatments in different samples containing bacteria and fungi. The authors stated that the low efficiency of Ag NPs in this study may be due to the presence of strains of mold that were unaffected by the Ag NPs.[18] Similar resistance was observed for the bacteria obtained from pre-Columbian archaeological cotton textiles compared with those from laboratory strains.

Becerra *et al.* observed that the performance of Ag NPs and the Ag NPs/TiO$_2$ hybrid against the formation of a biopatina on stone could be further improved by stabilizing the nanomaterials with the citrate ligand. These authors reported that, for a given initial quantity of Ag NPs, the ΔE is directly related to the aggregation of the nanoparticles: the more they tend to aggregate (zeta potential closer to 0 and higher hydrodynamic diameter) the higher the ΔE. The presence of citrate increases the zeta-potential while decreasing the hydrodynamic diameter, which avoids aggregation, while the presence of TiO$_2$ has the opposite effect.[21]

Another important factor on the effectiveness of treatments is the pH of the nanoparticle dispersion, as reported by Noeiaghaei *et al*, who demonstrated that the pH defined the stability of ZnO and Ag NPs against aggregation. For example, the same treatment of ZnO nanoparticles on a partially deteriorated mortar caused a 55-59% growth inhibition of *E. coli* over the pH range 6-10 *vs.* a 100% inhibition at pH 12; while for *B. cereus* the efficiency ranged 25-31% at pH in the range of 6-10 *vs.* 12% inhibition at pH 12.[65]

This clearly indicates the importance of the testing conditions on the antimicrobial performance of the treatments and emphasizes the need to study the native microorganisms present in the heritage objects as opposed to the more commonly studied strains.

ZnO NPs

The photocatalytic properties of ZnO are similar to those of TiO$_2$, with the advantage that ZnO is also an active antimicrobial agent under dark conditions. This is due to the toxic effect of zinc ions released into the local environment and/or from ZnO nanoparticles internalization by the microorganisms.[103] Gómez-Ortiz *et al.* successfully tested the antimicrobial features of pure ZnO and ZnO/Ca(OH)$_2$ hybrids on limestone in both dark and light conditions [58] and they also tried coatings containing Ca[Zn(OH)$_3$]·2H$_2$O that inhibited the growth of *A. niger* and *P. oxalicum*.[104] Other test case with calcium zinc hydroxide or ZnO is the work of Soria-Castro *et al.*, (2019) who affirm that the tested ZnO and [Ca(Zn(OH)$_3$)$_2$·2H$_2$O] NPs showed good activity against *S. aureus, E. coli, A. niger, P. oxalicum,* and *C. albicans*.

ZnO can completely inhibit the fungal growth, as reported for *A. niger* on stone (Figure 8a),[19,27,53] *Trichoderma viride* on paper[54] and *A. niger*, *A. versicolor* and *Rhizopus solani* on paper.[53] The efficiency of the treatment was increased under UV light (Figure 8b).[54] Ditaranto *et al.* reported a complete inhibition of *A. niger* growth in *in vitro* assays, although the corresponding *in situ* assay on stone did not provide significant results because, after six months, no growth was observed even in the control sample.[63] Other authors reported the inhibition of fungal growth in the range of 13-68% for four types of fungus (*A. alternata*, *A. niger*, *P. chrysogenum* and *Penicillium pinophilum*) isolated from an Egyptian tomb at the Valley of the Kings[25] 89.7% for *P. chrysogenum* isolated from an archaeological manuscript deposited at Al-Azhar Library of Cairo, Egypt,[49] 98.2% for *A. niger*

isolated from the book "Descriptio de L´Egypte", deposited at Misr library, Mansoura city, Egypt[83] and 97% for *C. Albicans* and 7% for *A. niger* on paper works.[22] A comprehensive publication by El-Feky *et al.* reported a reduction in the growth of *Trichoderma reesei* and *A. niger* on fresh oil paintings on paper and the reduction of the accumulation of dirt when left in the open air for six months (See Figure 8c).

ZnO has also been reported to completely inhibit the growth of *B. subtilis* and *P. chrysogenum* on paper (these strains were isolated from a manuscript from XVII century deposited at Al-Azhar Library, Cairo, Egypt)[49] and inhibit the growth of *S. aureus* and *E. coli* by 98.7 and 94.2% also on paper.[53] ZnO also delivers a 50% reduction on the growth of the algae *C. vulgaris* and *Scenedesmus quadricauda* on adobe mud and earthen artworks within 1 week (Figure 8d)[55] and presents an anti-fouling capacity close to 70% reduction on limestone.[61] In-doped ZnO can also reduce the growth of algae on stone.[55] Moreover, ZnO can provide additional features such as self-cleaning properties, UV-light protection[46] or structural reinforcement:[83] ageing tests with UV light for 150 h showed a reduction of color fading in samples treated with ZnO, and no appreciable change in the tensile strength of the nanoparticle-treated paper.[46]

Gambino *et al.* reported a dose-response effect of ZnO against *A. niger* similar to a hormetic behavior: a ZnO low-dose (0.25%) led to the accelerated sporulation, an earlier production of secondary metabolites and a change in the appearance of the biofilm, while a higher dose (0.5%) inhibited the fungal growth.[25] This phenomenon must be considered when designing an antimicrobial treatment due to the important consequences on the conservation of the heritage items. Recently, Schifani *et al.* have used ZnO nanorods supported on graphene to reduce the growth (60-90%) of the bacteria *Arthrobacter aurescens* and *Achromobacter spanius* on Noto stone, Carrara marble and bricks. Those strains were previously isolated from the Temple of the Concordia (Italy).[24]

SiO₂ NPs

The previous antimicrobial studies on these nanoparticles have focused on hybrids of SiO$_2$ NPs with photocatalytic materials based on TiO$_2$ and ZnO, organic biocides or biocidal metals or nanoparticles, such as Ag NPs. These mixtures are of particular interest in the field of heritage conservation because, besides their antimicrobial features, they also possess consolidating and hydrophobic properties.[45,57] Zarzuela *et al.*[45] developed silica-based nanocomposites with copper (II) oxide, *via* sol-gel route, as a multifunctional treatment in the protection of building stone. The compound, applied by impregnation to natural limestone, improves the mechanical resistance of the stone and reduces the growth of *E. coli* and *Saccharomyces cerevisiae*. Compared to the control samples, they obtained the highest inhibition with 87% for *E. coli* and 80% for *S. cerevisiae*, according to the CFU data of the cell recovery assay, with the compound containing a proportion of 0.15% w/v of CuO.

In other cases, the biocidal activity of Ag/SiO$_2$ NPs hybrids grafted into a modified silicate matrix on xerogel samples has been evaluated.[57] These hybrids were applied on four stone samples of different origin: Lecce Stone from the Salento region (Italy), granite from Meis quarry (Pontevedra, Spain) and limestone from the Estepa quarries (Seville, Spain) and Cabra (Córdoba, Spain); noting that Ag/SiO$_2$ NPs products increased biocidal effect by up to >90%, typically achieving *ca.* 80-98% bacterial inhibition against *E. coli* and *S. cerevisiae*.

Cu and CuO NPs

The bioactivity of Cu NPs against microorganisms relies on the formation of ROS, which cause multiple toxic effects including membrane lipis lipid peroxidation and degradation, protein oxidation or the degradation of DNA.[79] Despite the known antimicrobial surface properties and widespread commercial use of Cu and CuO NPs in the biomedical field, they have been relatively underexploited in heritage conservation, particularly when compared to other common antibacterial agents such as TiO$_2$ or Ag NPs. In this respect, one of the inherent limitations of copper is its rapid oxidation and its possible nanotoxicity. However, the available literature affirms that Cu NPs applied together with consolidants are highly effective in the treatment of stone surfaces colonized by bacteria, fungi, algae or lichens.[15,50]

Following an eight-year-long study, Pinna *et al.* (2018) evaluated the effectiveness of Cu NPs, together with several commercial consolidants and water-repellents (Silo™ 111, Acrilico™ 30 and Estel™ 1000), after their application to pre-cleaned substrates (sandstone, marble and plaster), from the archaeological site Area of Fiesole (Florence, Italy).[15] The authors concluded that the recolonization of the substrates after the treatment applied in 2008 was related to their bioreceptivity and the weather conditions. In this case, although the applied materials reduced colonization, after two-to-three years they failed to prevent the reappearance of lichens and biofilms.

The biogenic synthesis of CuO NPs from *E. coli* Z1 has been reported, including a study of the antimicrobial activity of Cu NPs and CuSO$_4$ solution against bacterial and fungal strains.[50] These nanoparticles were also suspended in two commercial consolidation polymers (Primal™ AC33 and a silicon polymer), to be applied on stone, where *E. coli, S. parvulus and B. subtilis* was reduced by between 61 and 68%. Helmi *et al.* also reported a promising antimicrobial activity of CuO NPs, comparable to that of Ag NPs, against strains of bacterial and fungi isolated from funeral masks from Saqqara necropolis (Egypt).[26] However, these authors recommend using Ag NPs for heritage-related applications instead of CuO, due to the stronger color variation of the latter.

MgO NPs

15

Nano-MgO is known to possess important antimicrobial activity due to the small particle size and a larger surface area, which lead to the increased generation of superoxide radicals and the increase in pH associated with the hydration of MgO. Both the radicals and high pH damage the cell membrane and induce lysis, which, after the loss of the intracellular content, lead to the death of the microorganisms.[105] In addition to combating microbial biodeterioration, MgO NPs can be used in other restoration processes, such as in the deacidification of paper or canvases.[106,107]

Typically, MgO or $Mg(OH)_2$ NPs are mixed with other nanomaterials acting as supports, such as hydroxyethyl cellulose (HEC) or hydroxypropyl cellulose (Klucel™ E), and the hybrids are subsequently applied to heritage materials such as paper or stone. For example, the antifungal activity of MgO NPs and $Mg(OH)_2$ NPs with hydroxypropyl cellulose, on cotton paper sheets (Rotilabo®, Carl Roth) was evaluated by introducing samples into a growth medium containing *S. cerevisiae* and *E. coli*.[56] The treated samples showed wider zones of inhibition than others treated with non-nanosized MgO. MgO NPs only presented biocidal activity against fungi, while $Mg(OH)_2$ NPs showed antifungal and antibacterial activity. The authors concluded that nanometer-sized MgO particle were more effective than non-nanosized particles due to better penetrability between paper fibres and that the use of hydroxypropyl cellulose provides an improved surface coating of paper.

Recently, Franco-Castillo *et al.* evaluated the use of 12-nm diameter nano-MgO, prepared using a simple sol-gel synthesis, on three samples of 18th century paper from the Archives of the Royal Botanical Gardens (Madrid, Spain). MgO NPs were shown to be bactericidal to both the Gram-negative and Gram-positive species at low concentrations (1.5 mg/mL for *E. coli* and 0.75 mg/mL for *B. subtilis*). This antibacterial effect was verified using both cell proliferation assays and colorimetric resazurin cell viability tests. An *in situ* assay based on a chromogenic agar was used to determine the activity of the nano-MgO coating over the papers, which illustrated how the nano-MgO-treated paper samples remained free of microbial colonisation (Figure 9). The low cytotoxicity of the particles to eukaryotic cells was also confirmed.[10] A subsequent article by the same team showed that the same MgO NPs possessed fungicidal properties against the fungi *A. niger*, *T. reesei* and *C. cladosporioides in vitro*. When applied to original heritage paper samples, the particles retained their fungicidal properties against two of the three molds tested. Microscopy inspection of the paper samples showed how the untreated paper samples were fully colonized by fungal mycelium, while the coated paper samples remained free from colonization (Figure 9). Further assays were carried out to determine the ability of the MgO NPs to inhibit the cellulase enzyme activity of *A. niger* and *T. reesei*. These assays demonstrated how the MgO NPs inactivate the cellulases in both fungi at concentrations below the fungicidal concentration, making the molds unable to degrade the cellulose of the paper to obtain their nutrients. It is important to remark that none of the concentrations used to protect the paper samples result in a color change of the paper ($\Delta E < 3$).[11]

Recently, Sierra-Fernandez *et al.* have also reported the use of MgO NPs and Zn-doped MgO NPs ($Mg_{1-x}Zn_xO$, x=0.096) as protective antifungal coatings for dolomitic and calcitic stones. The authors found that Zn-doping significantly improved the photocatalytic and antifungal capabilities of MgO and that $Mg_{1-x}Zn_xO$ nanoparticle-based treatment prevented microbial colonization on calcareous stone materials.[12] The MIC of the MgO NPs was determined to be 1.25 mg/mL for *A. niger* and *P. oxalicum*, and similarly, for the Zn-doped MgO NPs, 1.25 mg/mL for *A. niger* and 0.625 mg/mL for *P. oxalicum*. Regarding the stone samples treated with Zn-doped MgO, *A. niger* colonization decreased from 50.13 to 8.35% (on dolostone) and from 19.93 to 9.84% (on limestone); while the overall *P. oxalicum* colonization reduction was found to be 78.8% (on dolostone) and 88.2% (on limestone), compared to the untreated control substrates.

Other nanomaterials

Other nanomaterials recently tested as antimicrobial in heritage conservation include polyoxometalate-ionic liquids (POM-ILs), graphene oxide and polymer-nanoencapsulated essential oils. POM-ILs have recently gained attention due to the tailorable antimicrobial properties offered by the structural and compositional versatility of these materials. These ionic liquid materials (salts with a melting point below 100° C),[108] are a combination of nanoscale molecular metal-oxide anions (polyoxometalates) and bulky organic cations, typically alkylammonium or phosphonium cations. Importantly, both the POM anion and the organic cation can be independently tuned, offering access to a multifunctional materials library. The anticorrosive and antimicrobial properties of the POM-ILs along with their hydrophobicity make them highly suitable candidates for cultural heritage conservation. Importantly, broad-spectrum antimicrobial POM-IL materials can be obtained by modulating the chemical composition of the POM-IL, providing microbicidal activity in the µg/mL range against different bacterial and fungal strains. Furthermore, the application of a transparent coating which will not modify the aesthetic properties of the object make them applicable on heritage materials. Recently, Misra and co-workers have obtained successful results applying POM-ILs as a coating over different natural limestone samples of different porosity from the north-east France and Belgium. Two types of POM-ILs, POM-IL 1 ((n-$C_7H_{15})_4N)_8[\alpha$-$SiW_{11}O_{39}$] and POM-IL 2 ((n-$C_6H_{13})_3(C_{14}H_{29})N)_8[\alpha$-$SiW_{11}O_{39}$], which share identical POM structure with different counter-cations were used in this study. A 200 mg/mL solution (in acetone) of both POM-ILs was applied by brush as a protective coating over three types of stones - Belgian Blue (0,3% porosity), Romery (5% porosity) and Dom (30% porosity) - to protect them against simulated acid corrosion. The weight loss after the experiment shows how both POM-ILs are capable of protecting the integrity of the stones, especially for the porous Dom stone, where the weight loss after exposed to simulated acid-vapour corrosion was only 0.4% for the POM-IL 1 coated stone, while the uncoated Dom stone underwent complete loss of structure. These compounds were also tested against two bacterial strains, Gram-positive *B. subtilis* and Gram-negative *E. coli*. Both POM-ILs possessed bactericidal activity in solution at concentrations between 0.5 and

16

500 µg/mL, and a modified Japanese Industrial Standard (JIS Z 2801) analysis verified the surface bactericidal activity of the POM-ILs, reaching up to 100% bacterial reduction for the Belgian Blue stone. A new protocol using the chromogenic TBX agar was established to determine the biofilm prevention activity of the POM-ILs when applied as a coating (Figure 10). Those quantitative results were also commensurate with qualitative analysis by ESEM, fluorescence and confocal microscopy.[9] A recent study has demonstrated the antifungal activity of these POM-ILs against a mixed culture of molds (*Engyodontium album*, *Cladosporium cladosporioides*, *Alternaria alternata* and *Aspergillus fumigatus*) isolated from the surface of historical bricks from the Auschwitz II-Birkenau State Museum (Poland). These POM-ILs were applied as a coating on 19th century brick samples and then inoculated with the mixture of molds and incubated in a climate chamber for three weeks. The POM-IL coating showed very high antifungal activity, being able to completely inhibit the mold growth over the surface. ESEM analysis performed with the inoculated bricks demonstrated the toxic effect of the POM-ILs on the conidia.[109]

Regarding carbon nanomaterials, González-Dominguez *et al.* have recently patented the use of graphene oxide for preventing the colonization of ornamental rocks by lichens and moss.[110] Finally, Romano *et al.* have introduced the use of polymer-encapsulated essential oils (170 nm-diameter capsules) to eliminate the bacteria *E. coli* and *Kocuria rhizophila* from the red marble surface of an 18th century church altar.[111] Meanwhile, Saada *et al.* studied the effectiveness of a lemongrass oil nanoemulsion using Tween 20 as surfactant. Microorganisms isolated from a 9th century parchment were reduced by 100% (*Aspergillus fumigatus*) 98.7% (*Byssochlamys spectabilis*) 83.5% (*Cladosporium xanthochromaticum),* and 100% (*Streptomyces albidoflavus).*[112] Consequently, it is our opinion that micro and nanoencapsulated antimicrobial agents will be ever more present in future publications.

OVERVIEW OF BIOCHEMICAL TECHNIQUES FOR DETERMINING ANTIMICROBIAL AND ANTIBIOFILM PROPERTIES

Designing optimal antimicrobial agents to prevent biodeterioration of cultural heritages

Several key points must be deemed when designing antimicrobial agents to prevent biodeterioration of heritage materials. Even before antimicrobial activity is considered, one of the main issues is the chemical stability of the compound or nanomaterial being studied along with any possible adverse interaction with the heritage substrate material. For this reason, chemically stable agents are preferred. Importantly, the type of heritage material, its elemental composition and key properties, such as porosity, stability, and so on, should be carefully considered to prevent undesired interactions with the antimicrobial agent. As a brief example, metal oxide nanoparticles (e.g. TiO_2, ZnO, $Ca(OH)_2$) are, *a priori*, good candidates for the protection of porous stone, since they can become integrated into the physical structure of the substrate material (Figure 2); whereas others, such as Ag NPs, are less stable, more prone to oxidation, and release of metal ions that could induce unwanted damage to the stone in the long term. Furthermore, sustainable and reversible conservation practices must also be considered meaning that removal and reapplication are also desirable characteristics. We must also add to this consideration of generating safe and sustainable materials, that the use of antimicrobial agents that simultaneously possess low cyto- and ecocytotoxicity must be a priority; especially in outdoor environments and objects frequently manipulated by the restorers or the public.[30,44] As a result of our review, we can confirm that this is one of the key sustainable aspects that frequently goes unaddressed in the published literature and one which will come under increasing scrutiny.

Focussing solely on antimicrobial properties, there are two main ways in which a microbial community can be removed using chemical methods: by preventing proliferation of the microorganism (antimicrobial properties) or by inhibiting biofilm generation (antibiofilm properties) (Figure 11). When using an agent with antimicrobial properties, it is critically important to distinguish between bacteriostatic and bactericidal effect. While a *"bacteriostatic"* compound inhibits bacterial growth, a *"bactericidal"* agent will act to kill the bacteria. However, these two pure categories (bacteriostatic and bactericidal) only apply *in vitro*, and can be influenced by growth conditions, the initial bacterial inoculum, test duration, temperature of incubation, and multiple other conditions. Besides, bactericidal compounds are usually concentration-dependant, and there is required a minimum dose to exhibit the bactericidal effect.[113] The same categories can be attributed to antifungal compounds, those that inhibit the fungal growth will exhibit a *"fungistatic"* effect, and those that kill the fungi will exhibit a *"fungicidal effect"*. These categories are also influenced by the length of incubation, the medium, temperature, etc and only apply in *in vitro* studies.[114] In general, a bactericidal (or fungicidal) compound or material is preferred over one that exhibits a bacteriostatic (or fungistatic) effect, since the latter offers greater opportunity for the microorganism to mutate and create resistance to the compound, enabling a recolonization of the substrate material.[115] Besides these principal antimicrobial characteristics, the way each microbe interact with the substrate has to be studied, especially in the case of biodeterioration through chemical assimilation, where the microorganisms obtains their carbon source and energy from the substrate. In this special case, the addition of another property that inhibits or avoids the use of the substrate as the nourishment source could be preferable and complement a bacteriostatic (or fungistatic) effect. For example, MgO NPs, which require relatively high concentrations to prevent fungal growth (>1 mg/mL) also inhibit cellulase activity - the enzyme secreted by the molds to obtain glucose from the cellulose of the paper substrate - at sub- MIC concentrations, meaning that they effectively prevent the microbial colonization of heritage paper through bifunctional mode of action.[11]

In addition to the importance of considering the wide variety of characteristics of different classes of microorganisms, the protection offered by biofilms must also be considered when designing an antimicrobial treatment. Biofilms are defined as a community of microbial cells - interacting with one another and with the surface - embedded in a matrix of extracellular

polymeric substances.[116] The biofilm community can act to shield the microorganisms inside the matrix from the external aggressions, such as antimicrobials or harsh environmental conditions.[117] Therefore, the use of specifically designed antibiofilm agents is essential for combatting biodeterioration of any surface. There are two ways to tackle biofilm growth: i) preventing initial formation (or regrowth) or ii) removing a pre-existing biofilm. Nowadays, most antibiofilm compounds being developed are based on non-toxic molecules that do not directly affect the bacterial survival, to prevent future resistance to the compound.[117,118] These molecules can inhibit the microbial colonization on surfaces and the biofilm formation by regulating the expression of certain genes, inhibit the synthesis of the exopolymeric matrix or interrupting the molecular communication between bacteria - known as quorum sensing signals - that regulate the expression of a wide range of genes involved in virulence, antibiotic production, motility or biofilm formation, among others. Some examples of these molecules that are currently being used to prevent biofilm formation are indoles and their derivatives, peptides, D-aminoacids, free fatty acids, nitric oxide, ionic liquids[9] or quorum sensing inhibitors, among others. Other approaches to prevent biofilm formation consist of physical modification of the surface *via* 3D patterning or conferring new physicochemical properties such as hydrophobicity.[118] It is worth mentioning that, for some conservators, the microbial growth over a heritage surface (bio-patina) is considered as an integral part of the object. Consequently, there are cases where conservation and restoration methods should take this into consideration and attempt to preserve this patina.[119]

Some studies have reported on the synergistic effects promoted by antimicrobials in combination with antibiofilm agents. Darouiche and co-workers evaluated the combination of the antimicrobial triclosan with an antibiofilm enzyme against Gram-positive cocci and yeasts and demonstrated a synergistic activity between them, due to the increase of antimicrobial susceptibility induced by the antibiofilm enzyme.[120] This study is in agreement with previous reports, where the synergic effect of different antibiofilm agents in combination with antibiotics and non-antibiotic compounds was already reported.[121,122]

Applicable techniques for antimicrobial activity determination

In this section we summarize different techniques from the microbiology field that can be used to establish the antimicrobial properties of a compound or material. Determining the antimicrobial activity *in vitro* using rapid and accurate biochemical techniques is the first crucial step in evaluating and selecting the most appropriate antimicrobial agent to be used in real samples. Choosing the wrong assay or method could easily lead to false negative results (e.g. promising compounds may be mistakenly discarded) or false positive results (e.g. bacteriostatic compounds selected for aggressive microbial colonization). Furthermore, determining the antimicrobial activity with non-standardized assays makes it almost impossible to compare results between other reports in the literature. For all this, knowing how to select the right combination of assays to perform for different compounds or materials is required in order to obtain a true understanding of their activity and proceed with meaningful subsequent research.

Table 2 contains an overview of these techniques, the antimicrobial property information that can be extracted from the assays, the effect of the compound on the microorganisms, the mechanism of action of the technique as well as any inherent limitations. To facilitate the reading of the table some important definitions and criteria are discussed in this paragraph. Firstly, the minimum inhibitory concentration (MIC) is defined as the minimum concentration of an antimicrobial agent at which the growth of a microorganism stops. While the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) are both defined as the lowest concentration of an antimicrobial agent at which 99.9% of the final inoculum is killed.[123] In general, dilution methods - performed in liquid media - are preferred over diffusion methods - performed in agar-based solid media - to determine quantitative antimicrobial activities, like the MIC value. However, diffusion methods (Kirby-Bauer test or agar well diffusion test) are useful for screening of a large number of compounds and determine the susceptibility of the microorganism, since the bioactivity of each compound can be determined easily, cheaply and quickly. Most crucially of all, the solubility properties of a compound or material must be also taken into account when planning assays, since many water-insoluble compounds could lead to false negative activities if the aqueous culture media precipitates such materials from solution. In such cases new methods have been described, like the agar microdilution method, which allows the determination of the MIC of non-water-soluble compounds, like essential oils.[123,124] There are also specific methods for antifungal effects, like the poisoned food method, which provides information about the fungistatic effect of the compound against molds. Microbial viability and, consequently, the MCB, is usually determined by colorimetric assays, using chromogenic compounds as Resazurin[125] or XTT tetrazolium salt, which stain the bacterial cells and depends on their metabolic activity, allowing the differentiation between viable and non-viable bacteria. Fluorescence assays work in a similar way and can provide a more complete picture of the metabolic processes and the cell cycle using specialized equipment such as a flow cytometer or confocal and fluorescence microscope, however this requires access to expensive equipment and costly reagents as well as extensive user training. As additional information, the best and easiest method to confirm bacterial viability is the culture of the bacteria in solid media after performing the mentioned techniques. For further information about these techniques and other and more specific methods we highly recommend the recent review by Balouiri and co-workers on "Methods for *in vitro* evaluating antimicrobial activity: A review" (and relevant references therewithin).[123]

Table 2. *Methods used to determine the antimicrobial activity of a compound or nanomaterial.*

Technique	Antimicrobial property	Information obtained	Mechanism of action	Remarks of the technique

18

Agar (disk) diffusion test (Kirby-Bauer test)[123]	Inhibition of bacterial growth – Bacteriostatic effect	Antimicrobial susceptibility. Not distinguish between bacteriostatic and bactericide effect	Diffusion of the antimicrobial into de agar	Qualitative, not suitable for determining the MIC. The drug may not diffuse into de agar
Agar well diffusion test[123]	Inhibition of bacterial growth – Bacteriostatic effect	Antimicrobial activity of plant or microbial extracts.	Diffusion of the antimicrobial into de agar	Qualitative, not suitable for determining the MIC. The drug may not diffuse into de agar
Poisoned food method[123]	Inhibition of fungal growth – Fungistatic effect	Antifungal effect against molds	Inhibition of fungal growth over agar with the compound incorporated	Need to use a positive control (a known antimicrobial)
Broth dilution method[123]	Inhibition of microbial growth – Microbiostatic and Microbicide effect	MIC MBC can be obtained subculturing	Turbidity of the solution is proportional to the microbial growth	The compound can mask the microbial growth
Agar microdilution method[123,124]	Inhibition of microbial growth – Microbiostatic effect	MIC	Inhibition of microbial growth over agar with the compound incorporated	Preferred to the *"Broth method"* when having multiple isolates or if the compound mask the detection of growth
Time kill curve[123]	Bactericidal and fungicidal effect	MBC, MFC	This method reveals a time-dependent or concentration-dependent effect	This method can be used to determine the synergism of antagonism between two drugs
Resazurin/Alamar Blue[123,125]	Cell viability assay for prokaryotic and eukaryotic cells	MIC, MBC	Oxidation-reduction indicator	Change color from blue to pink in the presence of living organisms
XTT[123]	Cell viability assay for eukaryotic cells (fungal cells)	MBC	Quantification of metabolic activity of the cells	Allows the study of intact biofilms. Not suitable for comparison between different strains or species
Fluorescence[123,126]	Cell viability assay for prokaryotic and eukaryotic cells	MBC	Stains DNA. Discriminate between viable and dead cells based on membrane integrity (live/dead staining)	Avoids viable but nonculturable bacteria.[127] Expensive equipment required (Flow cytometer, Confocal or fluorescence Microscopy). Time consuming image processing

Specific methods used to determine the antibiofilm properties of an antimicrobial agent are described in Table 3, which defines the nature of the data obtained (quantitative or qualitative), the mechanism of action of the technique and the primary advantages and disadvantages. As for the antimicrobial activity, the biofilm can also be studied by colorimetric and fluorescence methods. Fluorescence staining provides quantitative and qualitative results, obtaining information also about the bacterial viability and can also provide information on mechanism of action, however it also requires access to expensive equipment, fully trained users, and data analysis is often time-consuming. On the other hand, the crystal violet assay (Gram's method) is an easy and inexpensive method to quantify biofilms, and does not require high-tech equipment; although it does not distinguish between viable and dead bacterial cells. Electron microscopy can provide qualitative information about the cell morphology and cell-wall integrity, which can be useful to determine the interaction between the cell and the compound. Besides, there are also specific assays for photosynthetic microorganisms, that enables the quantification of chlorophyll (the green pigments found in cyanobacteria, algae and plants, essential for photosynthesis) in a sample. Some of these methods are non-destructive and allow the study of the biofilms *in situ*. The metabolism of the cell can also be an indicator of biofilm mass and can be studied by ATP bioluminescence and fluorescein diacetate assays, but do not provide information about the cell morphology. For in-depth information concerning the most appropriate techniques for studying biofilms we recommend the comprehensive review by Wilson and co-workers *"Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review"* (and references therewithin).[128]

Table 3. *Methods to assess biofilm growth.*

Technique	Type	Mechanism of action	Advantages	Limitations
Fluorescence staining[128]	Quantitative and Qualitative	Stains the cells or the matrix of the	High resolution images, 3D images	Cost of the equipment (Confocal Scanning Laser

19

		biofilm with a fluorescent dye	Information about cellular viability, shape and function Information about spatial structures	Microscopy, Fluorescence Microscopy or Flow Cytometry) User training Use of specific and time-consuming software (e.g. Image J, COMSTAT)
Dry mass[128]	Quantitative - indirect	Biofilm quantification by the difference in weight between biomass on the substrate and the substrate with no biomass (achieved by temperature)	Inexpensive, easy and quick Low-tech equipment	Destructive The substrate must be heat resistant or easily separated from the biofilm
Crystal Violet[128]	Quantitative - indirect	Trianiline dye, cell membrane permeable in both gram + and gram - bacteria	Inexpensive, easy, reproducible and quick	Non-specific (does not distinguish between live and dead cells) Need for a standardized protocol
ATP bioluminescence[128]	Quantitative - indirect	Uses light (commonly produced by luciferase) to correlate the amount of ATP with the biofilm viability and biomass	Simple and quick Can be performed in both suspended or attached cells Availability of commercial kits Optional non-destructive assay with recombinant bacteria expressing GFP	Requires a luminometer, which must be calibrated regularly
Quartz crystal microbalance [128]	Quantitative - indirect	Uses the shift in resonance frequency due to microgram changes in mass to measure the biofilm accumulation as it is forming	Non-destructive measurement of the biofilm accumulation in real time Additional information about the viscoelastic properties can be obtained	Cost of the equipment, software and consumables Measurements are highly sensitive to changes in temperature and pressure
Chlorophyll a[129]	Quantitative - indirect	Quantification of the concentration of chlorophyll to estimate the amount of photosynthetic biomass	Allows measurement over the sample (without removing the biofilm)	Only suitable for photosynthetic microorganisms (e.g. algal biomass)
Fluorescein diacetate (FDA)[129]	Quantitative - indirect	Estimator of microbial biomass by measuring metabolic activity	Allows measurement over the sample (without removing the biofilm) Great sensitivity and rapid detection Suitable for non-scientific personnel with a minimum of scientific equipment	Enzymes released by damaged or inactive cells can overestimate the activity
Scanning Electron Microscopy[128]	Qualitative	Concentrated beam of electrons provides	High resolution images Possibility to perform elemental analysis (EDX)	Not suitable for living samples due to the high vacuum Sample preparation of living samples includes fixation,

20

| | | information about surfaces | | dehydration and sometimes coating with a conductive metal |
| Environmental Scanning Electron Microscopy[128] | Qualitative | Concentrated beam of electrons provides information about surfaces | No need for fixation process | Lower resolution than conventional SEM

The electron beam can harm the living sample if not fixed |

Monitoring biofilm growth *in situ* is an important goal relevant to the cultural heritage field. Biofilm formation and evolution can be studied *in situ* using the techniques in Table 4, however, some other more specific methods have been developed for specific substrate materials. For example, Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) spectroscopy allows the study of the biofilm at the molecular level, *in situ* and at real time, making it possible to study the evolution of the biofilm in response to environmental condition changes. Nonetheless, the biofilm must be grown over a specific ATR crystal with high refractive index to allow the detection of the infrared fingerprint of the sample, which may enable the study of the antibiofilm properties of a compound, but only applied on the ATR crystal and not applied on a real heritage sample or substrate material.[130]

Raman Spectroscopy can also be used to study biofilms, but suffers from the same limitations as the ART-FTIR method, in that it requires a specific substrate and does not allow the quantification of biofilm mass on real heritage samples. Furthermore, this technique frequently requires the use of colloidal metal nanoparticles (usually Ag or Au) to enhance the signal.[131,132]

With ESEM microscopy microbial growth over real samples can be studied, providing information about cell structure and morphology. However, this technique can only be applied on small laboratory samples (< approx. 5 cm in diameter).[128]

To the best of our knowledge, one of the best, most convenient and low-cost techniques to monitor biofilm growth *in situ* over a real heritage sample involves the determination of total color difference (ΔE^*). The total color difference is a non-destructive method that can be applied *in situ* and can be used to quantify the biofilm growth over real samples, as reported by *Prieto et al*. This method is easy to perform and does not require expensive equipment, only a colorimeter to measure the three values to obtain the total color difference (ΔE^*), the lightness (L^*) and the two chromaticity coordinates (a* (red-green); b* (yellow-blue). This color difference can be used as an indicator of microbial growth over the surface, enabling the quantification of the biofilm.[129] On the other hand, digital image analysis also allows the quantification of microbial colonization over different surfaces and this simple straightforward technique, can rapidly assess the growth of colored biofilms and microorganisms on heritage materials.[81,82]

CONCLUSIONS AND PERSPECTIVE

This multidisciplinary perspective review has been written to provide chemists, heritage scientists, and restoration and conservation professionals with a comprehensive overview on the use of antimicrobial nanomaterials for the conservation of cultural heritage objects and architectures. In summarizing the literature to date, we have strived to show how nanomaterials can provide alternatives to the traditional or commercial antimicrobial products used to prevent the biodeterioration of heritage objects by offering tailored and durable solutions for safeguarding different substrate materials. By addressing the question from a multidisciplinary standpoint, we hope that this review will be useful to readers initiating in the field as well as for expert readers who require a snapshot of the current state-of-the-art in order to provide cutting edge technological solutions for heritage conservation-restoration. Our conclusions and perspectives herein, give rise to several key challenges and opportunities for the area (summarized in Figure 12).

Breakthroughs leading to substantial leaps for the field will arise from combining multifunctional antimicrobial agents with advanced materials characterization to understand and engineer next-generation precision biocides that meet the particular needs of heritage conservation. Currently, however, there are still a limited number of examples where the fundamental physico-chemical properties of the nanomaterial can be adapted or improved to function without significantly altering the integrity of the object of interest. Herein lies the grand challenge for chemists and materials scientists: to chemically tailor nanomaterials to target specific microorganisms for particular end-uses in the conservation-restoration field, e.g. conservation of heritage items; removal of biofilms (or indeed prevent their formation); paralyze the formation of the bio-patina; avoid microbially influenced corrosion of metals and glass; or purify the air in museum display cases.

Over the last five years or so, there has been a clear move towards developing multifunctional coatings, where consolidation, antimicrobial, and water repellence properties, amongst others, are combined into one material. In our opinion, the key to success lies in developing libraries of such materials, but with the perspective of the conservator-restorer in mind. Moreover, the design of such antimicrobial agents should aim to meet the needs of the local environment (e.g. effect of ambient temperature, rainfall, humidity, UV exposure, and so on) and properly consider desired mode of action (i.e. either durable and long-lasting or highly active over a short period). This will permit researchers to develop materials that: i) target specific microorganisms responsible for deterioration and degradation of the heritage material, for example, cellulase-producing

bacteria and fungi (wood and paper biodeteriogens), or cyanobacteria and algae (the pioneering autotrophic colonizers of outdoor heritage), and so on; ii) function by direct application (e.g. as hydrophobic, antimicrobial or anticorrosive protective coatings), and iii) function indirectly (e.g. incorporated into display case filters or used as biosensors) to provide non-contact approaches to prevent microbial colonization.

Our analysis of the available literature on antimicrobial nanomaterials confirmed our premise that a variety of different classes of nanomaterials are being used to prevent biodeterioration of cultural heritage objects. Yet, few can be applied to a broad range of heritage surfaces or provide long-term antimicrobial action, essentially eliminating the potential for commercial application. It is patently clear that the time continuity of the projects is an issue and it is necessary to evaluate the performance of the antimicrobial treatments over longer periods of time, for instance from five-to-ten years, or longer. In this respect, a more frequent use of climate chamber tests for coatings and treatments would be a significant step forward, however it is also clear that studies should ideally be performed in real-world *in situ* settings (outdoors, in archives, in display cases, etc.), where anthropogenic conditions can be evaluated properly. The duration of research projects should consider the time frames that are necessary for the implementation of medium-to-long-term *in situ* evaluation through stable, collaborative programs involving museums, conservation-restoration professionals and research centres.

Such studies should be limited not only to the effectiveness of the treatment as antimicrobial, but also the whole process of designing, applying, testing the temporal evolution of the treatment - detachment, leaching or ions release - and the final disposing (when applicable): i.e., a holistic approach for the design of antimicrobials for the protection of cultural heritage items. Furthermore, one of the inherent disadvantages of many antimicrobial agents is that their bioactivity is frequently non-specific and so the cytotoxicity of such agents should be more completely evaluated. For example, Ag NPs and materials based on quaternary ammonium or phosphonium compounds are all highly cytotoxic and therefore have corresponding health, safety and environmental implications for restorers-conservators, curators and general public. In addition, ecotoxicological aspects should also be considered to comprehensively assess the release of nanoparticles (or products of degradation) during deposition or preparation of the coatings, during the coating lifetime. Finally, protocols for their removal or disposal should also be considered, since any such application should of course be reversible to facilitate its removal from the heritage surface, in line with conservation guidelines. Globally, these key considerations would more strictly adhere to the sustainable conservation principles that underpin the principal goals of the Heritage Science community at the present moment.

The literature illustrates how a combination of antimicrobial procedures together with advanced materials characterization techniques can be used to comprehensively evaluate the properties of different nanomaterials against biodeterioration. Consequently, a thorough understanding of the biodeterioration process and choice of appropriate microbiological assays is imperative. Our review of the literature leads us to emphasize the need for more precise evaluation of the antimicrobial properties of the materials being reported; with a specific need to identify more bactericidal and fungicidal agents, as opposed to bacteriostatic or fungistatic materials (which covers the vast majority of materials published to date). This characteristic affects the potential use of any antimicrobial agent and it is our view that there is a need to clearly distinguish between bacteriostatic, bactericide and antibiofilm so that it can be considered appropriately for the proposed end use. It is therefore important that the heritage science and biodeterioration community should work to establish systematic, standardized microbiological approaches to properly address the design and evaluation of antimicrobials to protect and preserve heritage items.

Finally, the heritage science field offers challenging research opportunities for chemists and materials scientists aiming to safeguard our shared cultural heritage for generations to come. In this respect, there is an ongoing requirement to develop antimicrobial solutions for lesser studies heritage materials, such as ivory,[133] soft and hard tissues in mummies,[134] papyrus and palm tree leaves, textiles,[135] or pigments[136] in prehistoric paintings. What is more, contemporary artworks produced from plastic polymers, acrylic resins, other organic substrates and varnishes and other protective substances that act as a protective barriers also require significant attention in coming years.[137] In summary, a series of multidisciplinary challenges to more effectively preserve our past and present cultural heritage for generations to come.

Resource Availability

Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by Dr. Scott G. Mitchell (scott.mitchell@csic.es).

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SUPPLEMENTAL INFORMATION
22

Supplemental PDF file contains Table S1 with additional information on the images and studies reported in Figure 1 of this Review.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the writing of this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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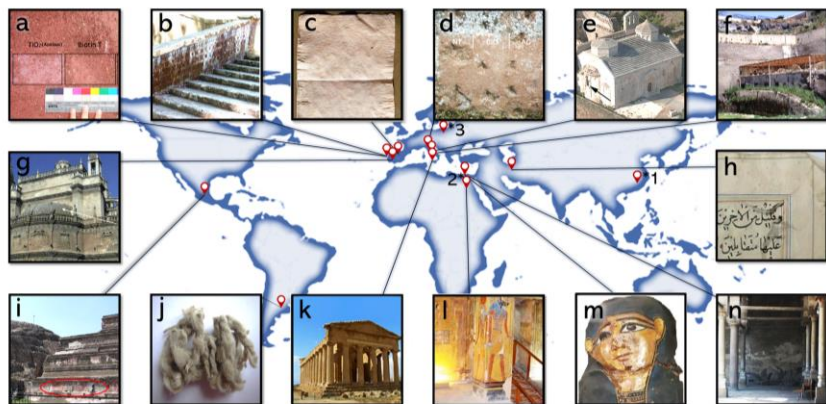
Figure 1. World Distribution and Heritage Items used in Antimicrobial Studies of Nanomaterials

(a) Walls on Palacio da Pena[14] and (b) glaze tiles at Marquis of Pombal Palace,[17] both in Portugal. (c) Paper sheet from the archive of the Royal Botanic Garden (Spain).[10] (d) Plaster wall from the Fiesole archeological area,[15] (e) Church of San Leonardo di Siponto[19] and (f) Villa dei papyri, all three from Italy.[20] (g) Cathedral of Seville (Spain).[21] (h) Old manuscript from Iran.[22] (i) Monument at Teotihuacán, Mexico.[16] (j) Pre-Colombian wool from La Plata Museum in Argentina.[23] (k) Temple of Concordia (Italy).[24] (l) Wall paintings at Valley of the Kings,[25] (m) funerary mask[26] and (n) columns from the Mosque of Amir Altinbugha al-Maridani,[27] all three from Egypt. All images were adapted with permission of the corresponding editorials. The references along with additional details are listed in Table 1 of the supplementary information. Note: *1, *2 and *3 denote additional heritage items whose picture has not been included. Please refer to Table S1 in the supplementary information for further details. a, d, f, g, h, I, j, l adapted from (see ref.) and reprinted with permission of Elsevier Masson SAS. Copyright © all rights reserved; b adapted from (see ref.) and reproduced with permission of John Wiley and Sons Copyright © all rights reserved; e, n adapted from (see ref.), Springer Nature, under creative commons license CC BY; k adapted from (see ref.) and reproduced under creative commons license CC BY; m adapted from (see ref.), and reproduced with permission of the editor, © Mediterranean Archaeology and Archaeometry, all rights reserved.
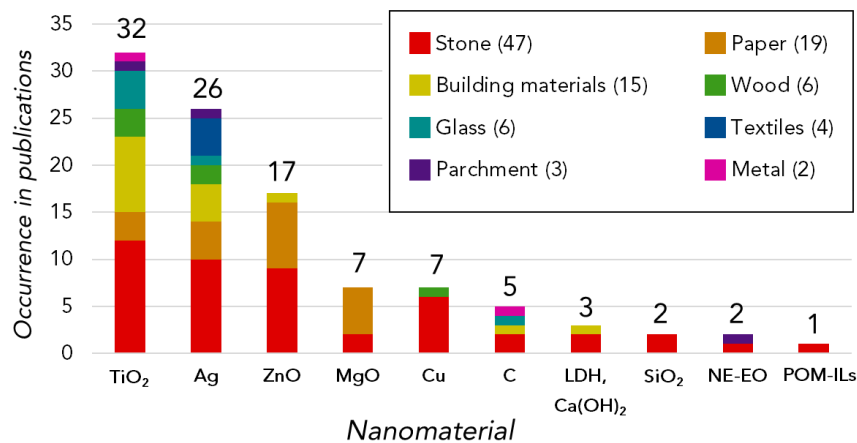
Figure 2. Frequency of Studies of a Nanomaterial for each Type of Heritage Item Substrate

Legend: C denotes carbon nanomaterials (graphene, graphene oxide and fullerenes); NE-EO, nano-encapsulated essential oils; and POM-ILs, polyoxometalate ionic-liquids. For further clarity, similar heritage materials have been grouped: Stone includes limestone, marble, calcareous stone and a fossil. Textiles include canvas and fabrics such as silk, wool and cotton. Building materials include a miscellanea of materials obtained from the transformation of inorganic sources used in construction and usually located in the outer side of buildings: bricks, ceramic tiles, plaster, adobe and mortar. Oil painting over paper has been included in paper, since the article refers mainly to the support and not the pigments. It must be highlighted here that some of the publications compare more than one nanomaterial or apply them over more than one type of substrates, which has been considered to the elaboration of this graph. Journal articles were acquired by searching for the keywords "antimicrobial", "bactericidal, "fungicidal", "antibacterial", "antifungal", "biocidal", "biodeterioration", "nanoparticles", "cultural heritage", "relics" and "monuments".
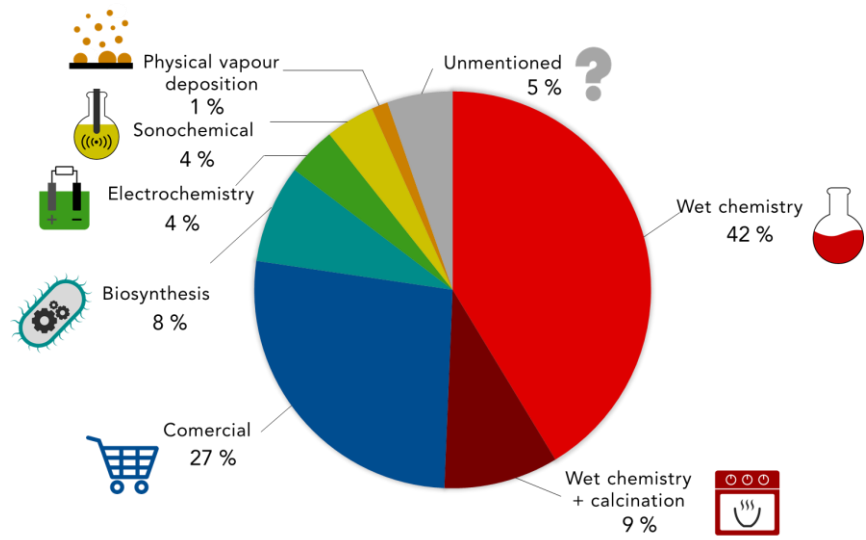
Figure 3. Origin of the Antimicrobial Nanomaterials used in Heritage Conservation
The graph includes chemical, physical and biological synthetic methods, commercial sources and those of unspecified origin.
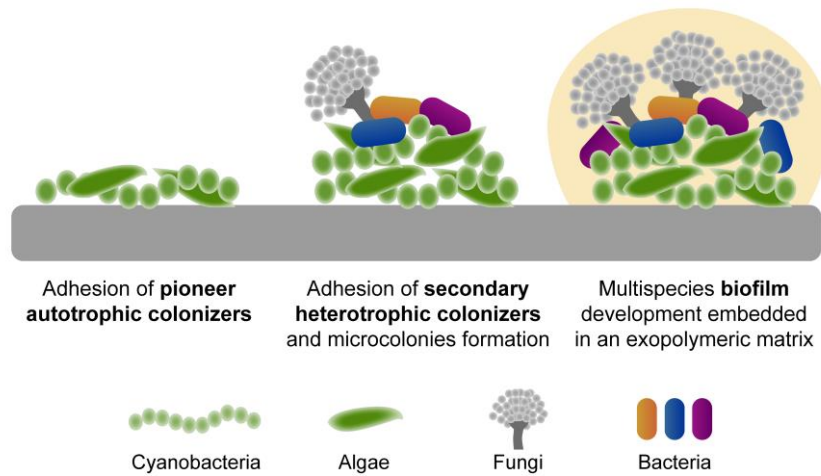
Figure 4. Outdoor Stone Surface Colonization Process

First, pioneering autotrophic colonizers (cyanobacteria and algae) attach to the surface, facilitating colonization by secondary heterotrophic colonizers (typically fungi and bacteria). Thereafter, multispecies biofilm development begins with the formation of microcolonies composed of different microorganisms which then evolves into a mature biofilm as the microorganisms become embedded in the matrix formed from the excretion of extracellular polymeric substances.
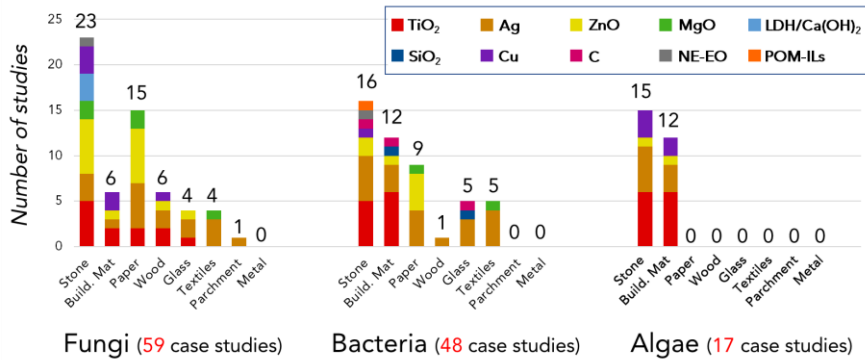
Figure 5. Frequency of Studies for Bacteria, Fungi and Algae

Legend: C, carbon nanomaterials (graphene, graphene oxide, fullerene); NE-EO, nano-encapsulated essential oils; POM-ILs, polyoxometalate Ionic-liquids. The studies are groups for type of substrate of cultural heritage. Each column details the number of studies per nanomaterial, in different colors (see legend).
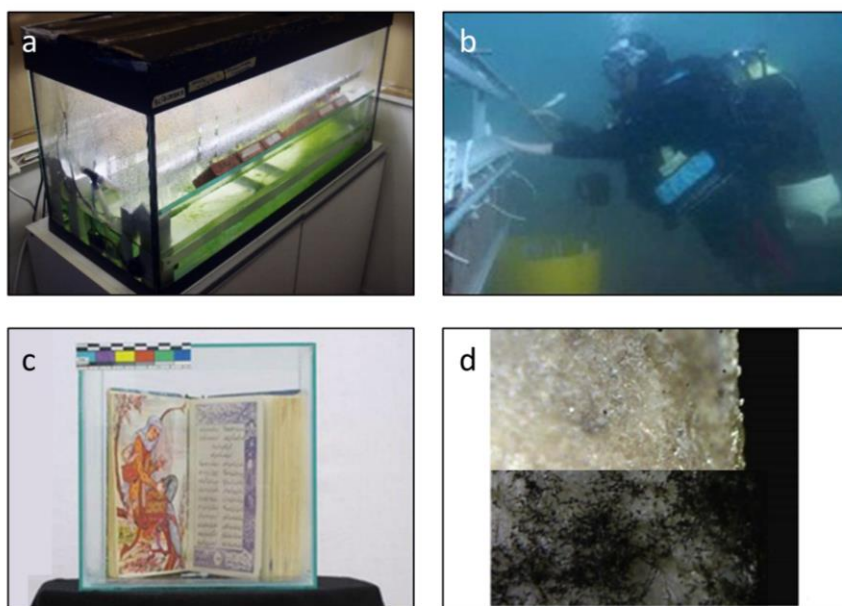
Figure 6. Images taken from studies of TiO₂ NPs as Antimicrobial in Heritage Conservation

(a) Climatic chamber used in the accelerated algal growth test,[87] (b) positioning of TiO₂-coated marble specimens under marine underwater environment,[72] (c) picture of a book inside the TiO₂-coated glass box[66] and (d) parchment colonized by *Penicillium chrysogenum* after (upper side) and before (lower side) the cleaning with TiO₂ in gellan gum.[48] a adapted from (see ref.) and reprinted with permission of MDPI under Creative Commons license CC BY (MDPI Editorial); b, c and d adapted from (see ref.) and reproduced with permission of Elsevier Masson SAS, Copyright © all rights reserved.
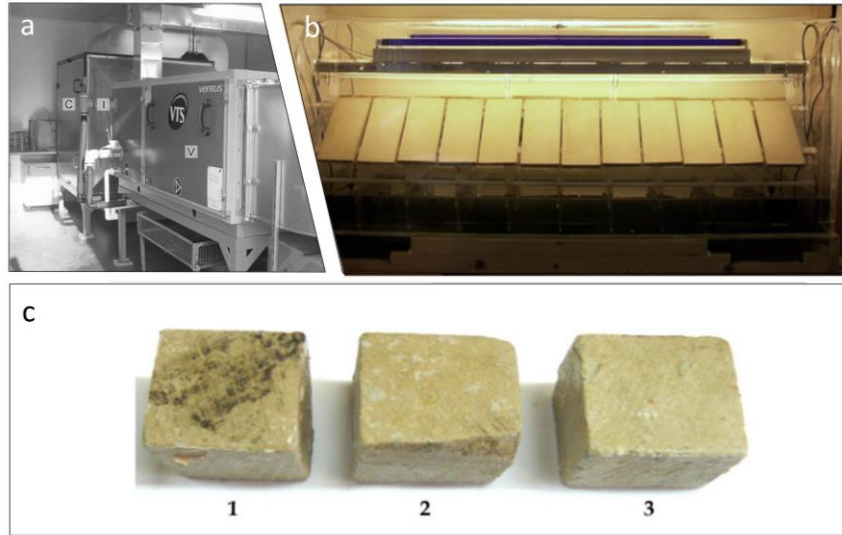
Figure 7. Images illustrating the Studies of Ag NPs as Antimicrobial in Heritage Conservation

(a) Misting chamber used for Ag NPs disinfection,[100] (b) culture streaming tank,[73] and (c) limestone blocks treated with a silicon polymer loaded with Ag NPs against *Aspergillus flavus*.[71] a, b and c adapted from (see ref.) and reproduced with permission of Elsevier Masson SAS. Copyright © all rights reserved.
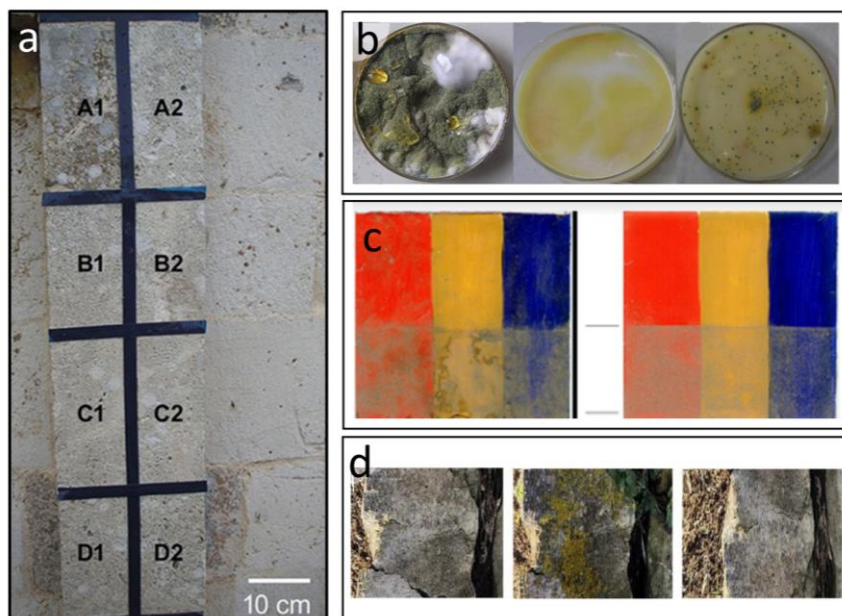
Figure 8. Images of the Studies of ZnO NPs as Antimicrobials in Heritage Conservation

(a) Test areas at the wall of the church of San Leonardo di Siponto (Italy). Zones C1 and C2 were cleaned with ZnO nanoparticles combined with commercial consolidants.[19] (b) Growth of mold on starch paste (left), and starch treated with ZnO under light (center) and in absence of light (right).[54] (c) Comparison of ZnO-treated oil paintings (upper part) and untreated (lower part) after six months of ageing (left side) and once cleaned with a brush (right side).[46] (d) Artwork surface original (left), stained by algae (center) and protected by ZnO nanoparticles (right).[55] a adapted from (see ref.), Springer Nature, under creative commons license CC BY; b adapted from (see ref.) and reproduced with permission of John Wiley and Sons. Copyright © all rights reserved; c and d adapted from (see ref.) and reproduced with permission of Elsevier Masson SAS. Copyright © all rights reserved.
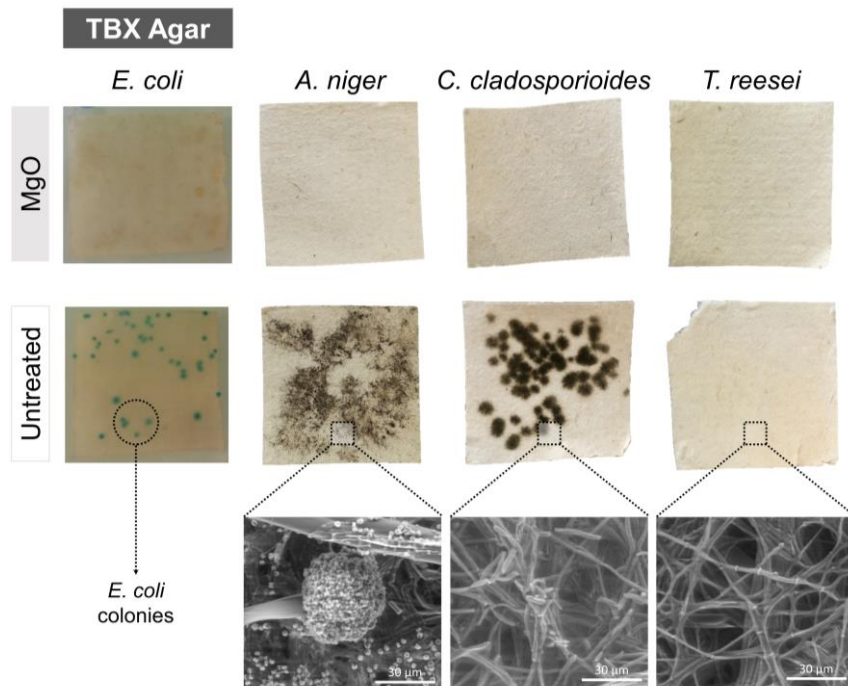
Figure 9. Use of Antimicrobial MgO NPs in Heritage Paper Conservation

Digital image analysis of 18th century paper samples (2 x 2 cm), coated with a 10 mg/mL MgO NPs solution and untreated control samples. The chromogenic TBX agar assay, performed with *E. coli*, reveals the growth inhibition properties of the MgO NPs (top left: absence of blue colonies of *E. coli*). MgO NPs also prevent fungal colonization of the paper samples, but visible fungal growth was evident in the untreated paper samples. ESEM imaging (below) confirmed the results.[10,11] Adapted and reproduced with permission of Elsevier Masson SAS and Royal Society of Chemistry (see ref.). Copyright © all rights reserved.
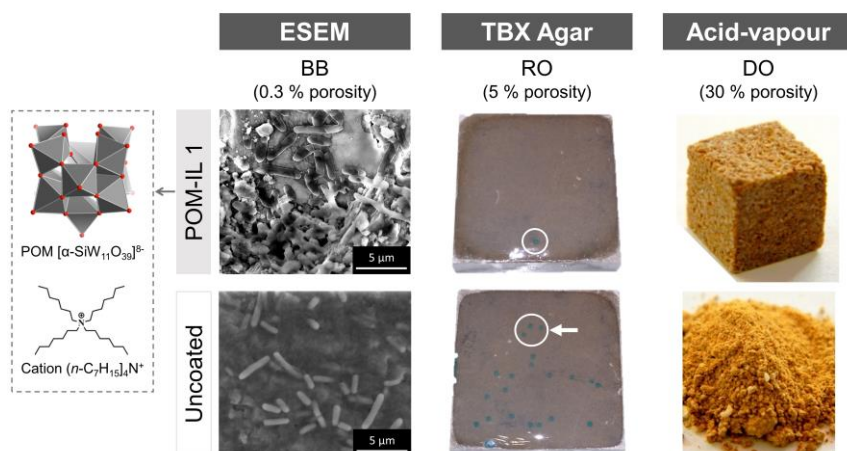
Figure 10. Antimicrobial and Anticorrosive POM-ILs in Heritage Conservation. ESEM, TBX Agar assay and acid-vapour test performed with the three different stones -Belgian Blue (BB), Romery (RO) and Dom (DO), respectively- coated with POM-IL 1 (images above) and without coating (images below). Both BB and DO stones, POM-IL 1 coated and uncoated, were inoculated with a solution of *E. coli*. As it can be observed in the ESEM results, bacteria incubated over the BB POM-IL 1 coated sample have lost their integrity, and present serious morphology damage, while in the uncoated stone they show a normal and healthy morphology. These results were commensurate with the TBX Agar assay performed with the RO stone. As in the ESEM assay, *E. coli* was in inoculated over a POM-IL 1 coated and uncoated RO stone and, after the incubation time, almost no growth was observed on the POM-IL 1 coated sample, while multiple colonies (blue spots) were found covering the uncoated stone. Furthermore, acid-vapour tests exposing the stone samples to acetic acid vapour for 72 h demonstrated the corrosion protection properties of the POM-ILs. This anticorrosive effect was particularly evident for the POM-IL 1 coating over the DO stone sample when compared with the uncoated control sample, which lost its structural integrity following the acid-vapour assay.[9] Adapted from (see ref.) and reproduced with permission of John Wiley and Sons. Copyright © all rights reserved.
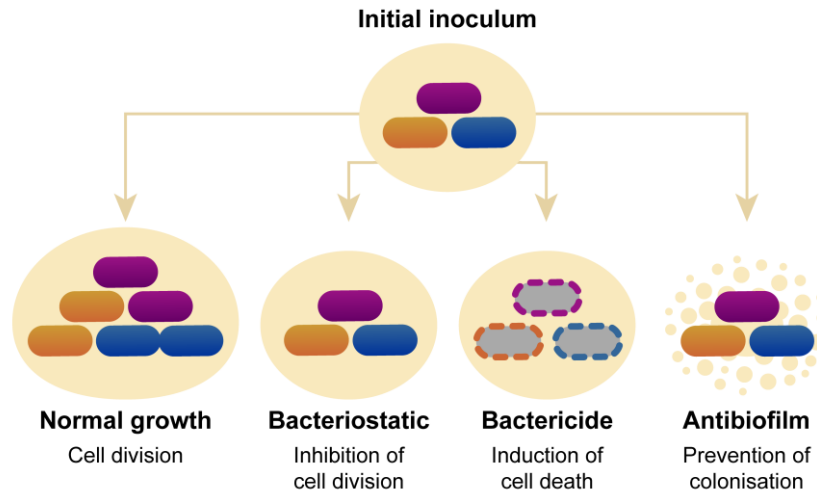
Figure 11. The effect of an antimicrobial agent to a bacterial inoculum. When the inoculum is not exposed to any drug, cell division occurs, and the culture grows. Bacteriostatic agents are capable of disable cell division, stopping bacterial growth, while bactericide agents kill the bacterial cells. Antibiofilm agents act by destroying the biofilm or preventing its formation.
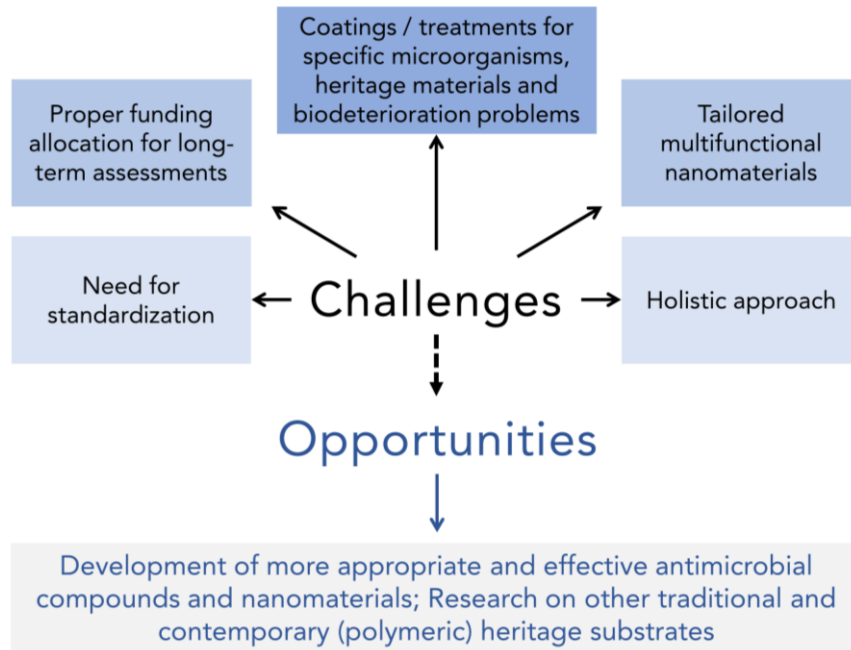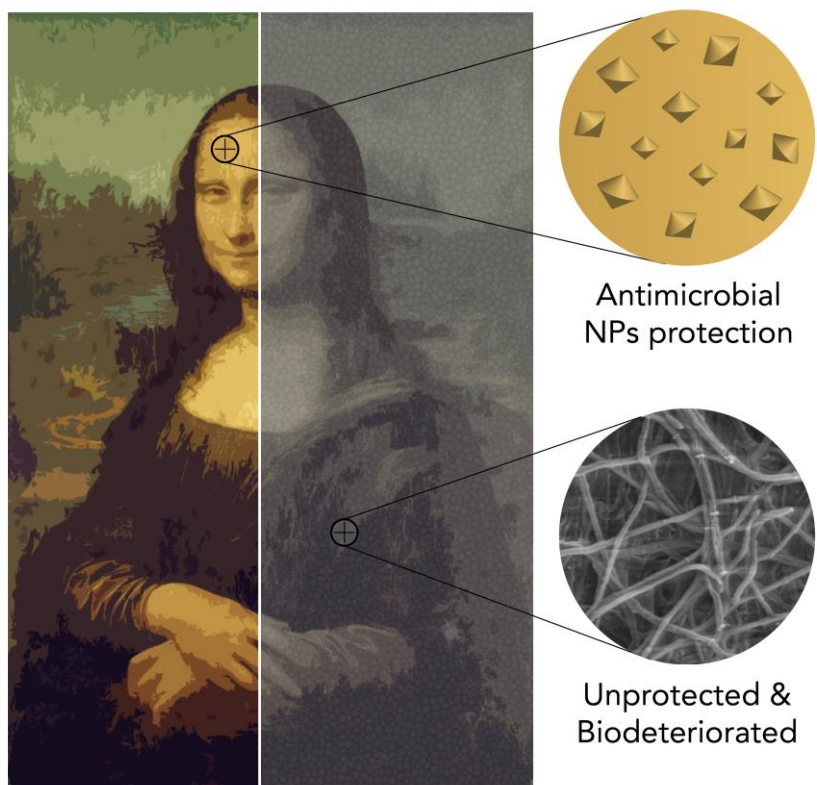
Figure 12. Challenges and opportunities in heritage conservation science.

Graphical abstract

The microbial deterioration of cultural heritage includes physical and chemical damage as well as aesthetic alteration. The aim of this multidisciplinary review is to provide a comprehensive outlook on the use of antimicrobial nanomaterials for the conservation of heritage objects and architectures. It offers practical advice from a restoration standpoint by comparing the state-of-the-art with antibacterial products used in heritage conservation, as well as guidance on selecting the most appropriate assays to correctly characterize antimicrobial activity.

Supplementary information:

Tests: (Col.) Colorimetry, (MC) microbial culture and (Ant) antimicrobial performance of the nanoparticles coatings.

	Tests			Heritage item	Location	City, country	Century	Ref.
Caption	Col.	M.C.	Ant.					
a	X		X	Arches yard walls, D. Carlos Terrace	Palacio da Pena	Sintra, Portugal	19th	[1]
b		X	X	Glazed wall tiles	Marquis of Pombal Palace	Oeiras, Portugal	18th	[2]
c	X		X	Paper sheet	Royal Botanic Gardens	Madrid, Spain	18th	[3]
d		X	X	Plaster, sandstone and marble	Archeological area of Fiesole	Firenze, Italy	3rd-1st BC	[4]
e	X			Limestone wall	San Leonardo di Siponto church	Manfredonia, Italy	12th	[5]
f		X	X	Plaster wall	Villa dei papiri	Ercolano, Naples, Italy	<1st	[6]
g		X		Vaults and walls	Cathedral and city hall	Seville, Spain	15th, 16th	[7]
h	X			Old manuscript	Unknown	Tehran, Iran	U	[8]
i		X		Stone wall	Archaeological monument at Teotihuacán	Mexico	<8th	[9]
j		X	X	Pre-columbian sisal, cotton & wool	La Plata Musseum	La Plata, Argentina	13th-15th	[10]
k		X		Temple of Concordia	Valley of the Temples	Agrigento, Italy	5th BC	[11]
l		X		Wall paintings	Tomb of Tausert and Setnakht, Valley of the Kings	Thebes, Egypt	12th BC	[12]
m		X		Egyptian funeral masks	Tomb	Saqqara, Egypt	22nd-3rd BC	[13]
n		X		Walls and columns	Al-Mansur Qalawun complex, Amr ibn al-As & Al-Tunbugha Al-Maridani mosques	Cairo, Egypt	7th, 13th & 14th	[14]
*1	X		X	Acidic book	U	China	U	[15]
*2		X		Book "Description de L´Egypte"	Misr library	Mansoura city, Egypt	19th	[16]
*2		X		Serh Senk Adlky Geld Badr manuscript	Al-Azhar library	Cairo, Egypt	18th	[17]
*3			X	-Paper map -Fragment of wooden floor from a church -Parchment (material for filling defects) -Canvas.	National Museum	Warsaw, Poland	18th/19th 17th U U	[18]
*3			X	Silk satin	Museum of Independence Tradition	Lodz, Poland	19th	[18]
*3		X	X	Book from Dziennik Ustaw, ed. 1924	Jozef Pilsudski library	Lodz, Poland	20th	[19]

*3		X	X	Book " Konsuelo", ed. 1985	National archive	Prague, Czech Republic.	19th	19
*3		X		Books, files Books Equipment, textiles, fabrics Paintings, paper, objects, surfaces Cabinets, sculptures, objects Furniture, objects	National archive Library Central museum of textiles Museum of Independence Tradition National museum Museum of Archeology and Ethnography	Lodz, Poland Lodz, Poland Lodz, Poland Lodz, Poland Warsaw, Poland Lodz, Poland	19th/20th 19th/20th 16th-20th 20th 13th-19th 19th/20th	20

Table 1. Tests (colorimetry, microbial culture and antimicrobial performance) performed in the heritage items listed in Figure X. The heritage items, their location, country and century are also described. U means *unknown*.

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