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Current knowledge regarding biological recolonization of stone cultural heritage after cleaning treatments

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ABSTRACT

Biocolonization causes physical-chemical and aesthetic biodeterioration, which depreciates the artistic value of outdoor works of art, leading to the use of (often expensive) treatments to remove the colonizing organisms. Such treatments are generally considered successful if they eliminate the biocolonization; however, subsequent recolonization of the cleaned substrate is generally overlooked by both public administrations and researchers. This review aimed to gather current scientific knowledge about the biological recolonization of stone-built cultural heritage after cleaning treatments. It is difficult to draw strong conclusions from the few studies on recolonization of cultural heritage, as each study involves different treatments, target organisms, substrates and climatic conditions. However, recolonization by fungi appears to be faster than recolonization by other organisms. Long term studies should be conducted to identify recolonization processes that may take some time and also involve various types of organisms. Short-term studies have only detected recolonization by generalist species, while long-term studies have shown recolonization by specialist species similar to the previous colonizer community.

1. Introduction

Living organisms are, together with water, among the main deterioration agents affecting stone-built cultural heritage. Numerous reviews of the biodeteriogenic character of heritage-colonizing organisms have been conducted [1–6]. In general, organisms that colonize stone substrates can cause physical and chemical deterioration of the materials on which they settle. Physical deterioration is caused by the expansion-contraction cycles that some of the organisms' structures undergo in contact with water [6,7] and chemical deterioration is caused by excretion of metabolic products (acids and chelates) that can react with the substrate 3,8[8]. Even when living organisms do not give rise to physical and/or chemical deterioration processes, they undoubtedly decrease the value of the heritage elements through aesthetic damage caused by the accumulation of biological material (biofouling), which disfigures or prevents full appreciation of the work of art (Fig. 1). Thus, some of the most important tasks in the restoration-conservation of cultural heritage are those involving cleaning and control of biological colonization. Indeed, a large part of the resources available for conservation-

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Fig. 1. Profuse biological colonization on granite works of art.

restoration of cultural heritage is allocated to such tasks. However, despite the efforts and resources used, biological colonization often reappears on the cleaned surfaces shortly after treatment [9–12]. In most cases, it is not known whether the recolonization is carried out by the pre-existing species or by other species.

Despite the importance of the recolonization process, both in the subsequent biodeterioration [13] and in relation to economic aspects, efforts to eliminate biological colonization and research studies conducted in this field generally only focus on “current” elimination without considering “potential” recolonization. Thus, most of the research aimed at controlling biological colonization only addresses the efficacy of cleaning treatments from the point of view of the level of cleaning achieved [14–16].

A bibliometric analysis including the terms “cultural heritage” OR monument* AND (clean* OR biocid* OR laser) returned a total of 3491 documents; among these, a keyword co-occurrence analysis, which took into account a minimum of 10 co-occurrences, established 70 items divided into 4 clusters (Fig. 2). In the figure, yellow, green and blue clusters include terms related to methodology in cultural heritage studies, while red clusters include those terms most closely related to stone-built cultural heritage and biological interactions and those referring to methods of cleaning or elimination of organisms, such as “laser cleaning”, “biocleaning”, “biocides”

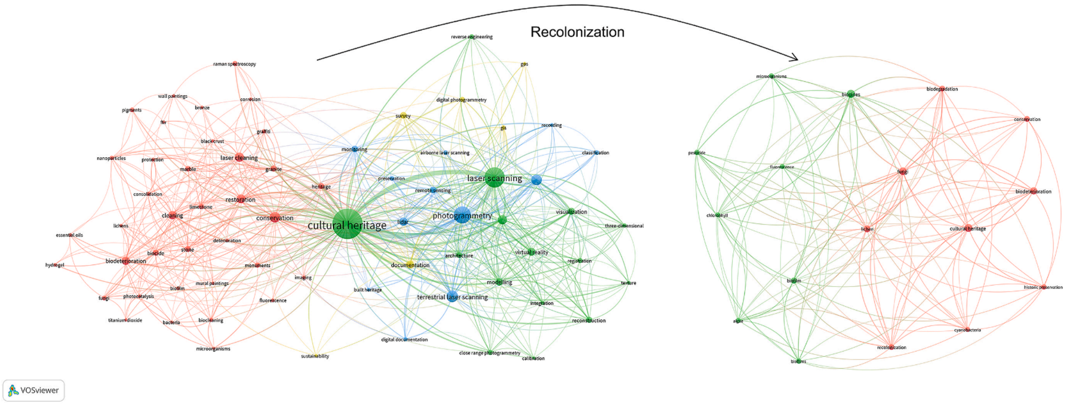

Fig. 2. Bibliometric analysis using VOSwiever software (Data source: SCOPUS).

and “essential oils”. Thus, although many studies address the cleaning of cultural heritage, only a third of them deal with the removal of biological colonization.

Including the word recolonization as a filter term in this set of references only returned 45 studies. Setting the minimum number of co-occurrences in this set of 45 articles to a value of 5, the keyword co-occurrence analysis returned only 17 keywords divided into two clusters, with the term “recolonization” in the same cluster (red) as “cultural heritage” and terms related to deterioration and conservation, but in a different cluster from the keywords referring to biological colonization removal treatments (Fig. 2). This indicates that research on recolonization of cultural heritage after cleaning protocols is scarce, and that the studies conducted to date mainly involve biodeterioration of cultural heritage sites. In addition, examination of the 45 studies returned by the database for the term recolonization confirmed that less than half of these deal with recolonization; the others only include the term because they indicate that recolonization studies are necessary, but they do not actually address the problem.

It should also be noted that, in the scientific field of cultural heritage conservation, many studies are published in conference proceedings, and the data from such studies are not always included in the databases.

2. Removal of biological colonization

Biodeteriogens can be removed from the surface of cultural heritage elements by physical or chemical (synthetic and natural) methods, most of which are used to remove all types of superficial particles (not only biodeteriogens). However, other methods, including the use of chemical products or specific types of radiation, have been specifically designed to affect vital aspects of the organisms.

Among the physical methods, the most commonly used (and studied) consists of the removal of organisms by gentle brushing of the colonized surface. Although this is a relatively simple task, there is no consensus regarding the method that should be used, since some authors and/or restorers brush only dry surfaces [17], others brush the surface after a (non-standardized) time of application of deionized water or biocides [18,19], others brush the surface as a prior step to applying biocides [20,21] and yet others combine dry and wet brushing [22].

Considering the efficacy of removal only as the level of cleaning achieved, Mascalchi et al. [18] indicate that mechanical cleaning by brushing is sufficient to remove the surface layer of lichens and biofilms from sandstone and marble gravestones, but insufficient to remove endolithic cells. Sanmartín et al. [19] consider this treatment to be effective for granite, but also indicate that for removal of algal biofilm there is no difference in the efficacy of brushing with water and brushing with a biocide.

In addition, brushing can damage the substrate [23] and facilitate the entry of propagules into deeper areas of the substrate [24] thus facilitating subsequent recolonization. Furthermore, it may give rise to the dispersal of propagules to the environment. A promising alternative to brushing before the application of biocides is the use of hydrogels. It has been demonstrated that hydrogels increase the contact between the products and the organisms and avoid the need to brush the surface, as the dead organisms are removed together with the hydrogel [25]. The application of the hydrogel alone without any added product, has been found to be sufficient to eliminate colonization owing to a physical effect favoured by the close contact with the biofilm [26].

The second most studied physical method, although not widely used in cleaning work for practical reasons, is the use of laser radiation [27–29]. This method is based on an ablation process involving thermal, photochemical and photomechanical mechanisms that depend on the type of laser, wavelength, pulse duration and fluence used.

The main advantages of the laser-based method are that the equipment does not come into contact with the object to be cleaned, the size of the beam can be regulated, thus enabling areas of different dimensions to be cleaned, and the process does not have adverse effects on the environment [30]. However, the method must be applied by authorized personnel as it involves radiation and leads to the release of particles from the substrate that could potentially be inhaled and cause diseases such as asbestosis and silicosis [31]. Another disadvantage is that the method must be optimized for use with different substrates to prevent damaging the material [30]. Most of the relevant studies on optimization processes have been carried out in the laboratory with industrial lasers, and adapting the methods for use with portable lasers is difficult.

The target organisms in most studies involving laser radiation cleaning are lichens, probably because they are difficult to remove by other methods. Thus, several studies have shown the effectiveness of IR (1604 nm) and UV (266 and 355 nm) laser radiation in removing heritage-colonizing lichens [14,20,32–35]. IR radiation (1064 nm) is known to produce ultrastructural changes and metabolic damage in the mycobiont, thus facilitating inactivation and removal of epilithic and endolytic colonizers [36]. By contrast, UV radiation (266 and 355 nm) induces rather superficial, albeit effective, damage in the lichen thallus, with a more pronounced effect at 266 nm than at 355 nm, probably due to greater absorption of shorter wavelengths [14,32]. However, although the level of cleaning or removal of lichens depends on the wavelength used [37], complete removal is not generally achieved [14,33,38]. Thus, although damage to the structure of the lichens weakens their adherence to the substrate, subsequent brushing is required to remove the organisms [20].

Another important aspect to be considered is the damage that laser radiation can cause to the substrate. Important levels of damage to polymineralic rocks due to thermal interaction between IR laser radiation and the substrate have been reported. Thus, the use of IR laser radiation to remove lichens from schist samples was found to cause grooves in the lithic substrate, leading to an increase in the substrate bioreceptivity [20], and in the case of basalt [39] and granite [37] resulted in the melting of dark minerals.

Microwave heating is a physical method that has recently been introduced as an environmentally friendly alternative to traditional methods of heritage conservation. The microwave radiation dissipates in the material and only causes heating if water is present, so that only living cells, and not the rock, are affected. As currently available devices only allow depths of to 15 mm to be reached, the method cannot be used to remove endolithic organisms [40]. In this type of treatment, it is essential to determine the temperature/time dose, which varies depending on the organism involved. Thus, the vitality of the lichen photobiont is affected by the heating to 50 °C for 3 min, while mycobiont requires heating to 65–70 °C, depending on the specimen [41].

The efficacy of heat treatments for the removal of epilithic and endolithic lichens and bryophytes has been demonstrated in both on-site and laboratory experiments [42,43]. The results confirmed that 6–12 h of treatment at 55–60 °C kills lichens and bryophytes as long as the organisms are fully hydrated. However, Bertuzzi et al. [44] pointed that although this type of heat treatment helps to eliminate some biodeteriogens and damages others, it may actually favour the development of surviving cells when applied to unicellular green algae.

Most recent studies indicate the usefulness of combining microwave treatment with other techniques. Thus, laser cleaning and microwave treatment could be used together to remove lichens. Microwave radiation can be applied after laser ablation treatment, or in some cases, e.g. where biodeteriogens do not absorb sufficient energy at 532 nm (e.g. organisms with chlorophyll as the predominant chromophore), microwave radiation can be applied before laser treatment, with the aim of damaging PSII and darkening the chlorophyll by inducing the formation of pheophytins, and thus increasing its absorption coefficient [45].

Another physical method consists of exposing organisms to UV-C light. Use of this method is almost exclusively restricted to caves due to the damaging effects of this light on living organisms. Use of this method to kill biological colonization agents is mainly based on the damage this type of light causes to the PSII photosystem, as demonstrated by Borderie et al. [46] for cyanobacteria and eukaryotic algae. However, its efficacy may be weakened by the low penetration power so that, in the case of thick biofilms, the cells in the lower layers may remain unaltered and resistant fungi may utilize dead matter (killed by the laser treatment) to recolonize the treated areas [47].

Regarding chemical methods, the most commonly used (and studied) is the application of biocides (e.g. Refs. [7,18–21,48–51]). Biocides are chemical compounds with antibacterial properties. Although many biocides are available, only a few can be used in preservation/restoration, including benzalkonium chloride, which dissociates the lipid bilayer of the cell membrane, and isothiazolones, which destroy proteins and DNA.

There is a high demand for this type of product, and there are numerous commercial biocides available on the market (e.g. Preventol RI 80, Biotin T, ROCIMA 103), each which has a different mechanism of action and toxic effect on organisms. Despite being the most studied type of heritage conservation treatment, and because of the commercial range of these products, studies on the same product applied to different organisms and substrates are very scarce and generally used different application methods. Thus, it is very difficult to establish any general trends regarding their efficacy, in practical cases of restoration, the products are usually selected on the basis of the experience of the restorers and/or of the expertise of the commercial companies in selling their products.

The articles published to date, especially those comparing the use of biocides with other methods of removing biological colonization, show that biocides are very effective. However, cases of biofilm resistance to biocides have already begun to be reported [52]. In some cases, degradation of the biofilms leads to the release of nitrogen and carbon, thereby favouring, rather than preventing, recolonization by other, sometimes more aggressive, species [53–55]. This process may lead to toxicity-related problems and associated environmental impacts such as those derived from the runoff of biocides from buildings facades. Biocides can be degraded through photolytic and hydrolytic processes, and the resulting products can reach the soil or receiving waters. In addition, the persistence of biocides in the environment depends on the physicochemical properties of both the substrate and the compounds, which can be very variable. In that context, Styszko et al., 2014 [56] evaluated the desorption constants of several biocides towards acrylate- and silicone-based renders and showed that the more lipophilic compounds, such as isothiazolones, are released much more slowly in the aqueous phase than the more hydrophilic compounds such as isoproturon and diuron, although the process is strongly influenced by the composition of the render. Furthermore, environmental conditions and the moisture content of the substrate can strongly influence leaching of biocides. Bollman 2016 and 2017 [57,58] found that time between precipitation events in cool temperate regions is shorter than the degradation half-life of biocides such as isothiazolones. In addition, Styszko 2015 [59] demonstrated that more leaching occurs from previously soaked substrates. Consequently, the residues of some biocides are likely to be present due to repeated input of water, and in this context most biocides can be considered “pseudopersistent” contaminants [57,58,60]. Most studies focus on the degradation mechanisms of biocides in surface water, which may differ from those occurring on the surface of buildings. Therefore, the degradation products of many of the biocides most commonly used on stone-built cultural heritage are currently not well known, although they are believed to be highly ecotoxic [61]. There has therefore been a tendency in recent years to search for natural biocides.

The use of essential oils is a possible natural alternative chemical method. Essential oils are secondary metabolites produced by plants as defence against pathogenic microorganisms and predators. They have proven biocidal properties, even at low concentrations not considered harmful to humans or the environment. Stupar et al. [53] tested the efficacy of some essential oils extracted from plants, especially those rich in phenolic compounds, to eliminate fungal strains naturally present on stone-built materials, relative to the efficacy of biocides based on quaternary ammonium salts. Mironescu and Georgescu [62] also reported that essential oils mainly composed of phenols were more effective as antifungal agents than those composed of hydrocarbons.

Regarding the potential use of these natural compounds to eliminate photoautotrophic biological colonization, Bruno et al. [63] tested the efficacy of essential oils extracted from *Lavandula angustifolia* and *Thymus vulgaris* to remove biofilms present in catacombs, observing that they were effective even at low concentrations, although two applications were required in this case.

The effect of secondary metabolites produced by saxicolous lichens has also been studied [64]. All secondary metabolites tested (usnic acid, norethic acid and parietin) effectively inhibited the development of fungi, cyanobacteria and green algae, in a similar way to benzalkonium chloride. These findings are very promising as they could be used to eliminate biofilm-forming organisms. In a very recent study, Cardellicchio et al. [16] evaluated the biocidal activity of a mixture of natural glycoalkaloids extracted from the unripe fruit of *Solanum nigrum* and used to clean the surface of a hypogeum wall covered by biofilm; the treatment was effective after about four weeks, for most biofilm colonizers but not for fungal species.

Thus, although various physical and chemical methods can successfully remove colonization, they have serious disadvantages as they can cause damage to the substrate, workers and/or the environment. Although physical methods involving radiation, such as those based on UV and microwave application, are respectful of the substrate and environment, careful measures must be taken to ensure that they do not affect people. Chemical methods seem to be more selective, but they are toxic to both people and the environment and also leave residues on the substrate.

3. Efficacy of removal treatments in preventing recolonization

Most of the treatments used to remove biological colonization can successfully eliminate the colonising organisms [9,18,65]; however, the question remains as to whether they are effective in preventing subsequent recolonization. The bibliometric analysis presented above showed that studies on the process of recolonization after treatment of heritage objects are scarce and very recent. This is probably due to the difficulty in conducting this kind of study as, in addition to the effect of the products applied and the method of application, the many factors affecting biocolonization must also be considered. Table 1 lists the few scientific articles (18) dealing with recolonization of stone-built cultural heritage after removal of biological colonization. Most of the studies involve the use of chemical biocides to remove lichen, cyanobacteria and algae and fewer studies involved fungi or bacteria. In addition, most of the studies have been carried out on carbonate rocks, and only two involve silicic rocks.

Studies on recolonization of stone-built gravestones initially colonized by lichens and biofilms and treated by brushing, microwave heating or with ROCIMA 103 biocide have been conducted by Mascalchi et al. [18]. They reported that recolonization was fastest in the case of brushing, it occurred after 15 months in the microwave-treated areas while only one of the areas treated with biocide was recolonized by fungi after 5 years. However, a waterproof coating (Silo 111) had been applied after all cleaning treatments, so that it is difficult to distinguish between the role of the cleaning treatment and that of the water repellent coating in the recolonization process. The study findings (earlier recolonization after microwave treatment) somewhat invalidate the hypothesis previously proposed by the same authors, according to which microwave heat treatment may delay recolonization [40]. The findings may confirm those of other authors [74], who indicated that caution should be exercised when using microwave heat treatment to remove unicellular green algae, as the treatment may favour growth of surviving cells.

Other studies dealing with lichen recolonization include those conducted by Paz-Bermúdez et al. [35] and Pozo-Antonio et al. [38] on shale from the Foz-Coa and Siega Verde archaeological parks and treated with Biotin-T, benzalkonium chloride biocides or laser radiation, and by Nascimbene and Salvadori [69] on limestone statues in parks in Venice treated by brushing and biocides, consolidants or water repellents. Biotin-T biocide and benzalkonium chloride produced better results than laser cleaning, in terms of preventing recolonization by lichens on shale as recolonization did not occur after 4 years. However, in the case of the limestone statues, recolonization began 6 months after treatment and became evident after 7 years and involved communities of only 4 species, which covered more than 50% of the area covered prior to the treatment. The study by Nascimbene and Salvadori [69] is one of the few studies considering, or at least indicating, that environmental conditions may determine differences in cover and diversity of recolonization. The results of this study, together with a later study on one of the statues [70], lead to a very interesting proposal by the authors, i.e. “ If restorations are not going to be maintained, it would be better to retain a more historic, diverse, and complex lichen community on artworks rather than a simplified community of “weedy” lichens that quickly cover almost the same area as the pre-restoration community did".

Some other examples can be given of recolonization involving the replacement of some organisms and species by others. Pfendler et al. [47] reported the replacement of phototrophs by fungi on the walls of caves treated by UV-C. As mentioned above, Bastian et al. [54], Martin-Sanchez et al. [55] and Stupar et al. [53] showed that the degradation of biocides and the death of organisms acted as sources of nitrogen and carbon, thus favouring recolonization by other, sometimes more aggressive, species. Urzì et al. [72] found that application of biocides to biofilms in catacombs resulted in colonization of the treated surfaces by bacteria. Hallmann et al. [67] observed differences in the composition of algal and fungal communities of biofilms before and one year after cleaning a marble sculpture. Finally, Zhu et al. [73] observed a considerable difference in the microbial species composition and community structures before and after treatment of a stone surface with a new blend of biocides, with the generalist species (Proteobacteria and Epistylidae) replacing the specialist species (Cyanobacteria, Chlorophyta and Ascomycetes).

In comparisons of cultural heritage artefacts with the same type of substrate and located in the same type of environment, climatic factors are particularly important in relation to biocolonization, along with the history and biogeographic characteristics of the site [75]. Climatic and microclimatic conditions, such as humidity, temperature and light, undoubtedly determine whether a substrate will be recolonized, and even small changes in microclimatic conditions can create unsuitable conditions for the development of biodeteriogens [76]. Thus, controlling climatic and microclimatic conditions, as well as controlling the dispersion of biological propagules, may be the best way of preventing recolonization. Morando et al. [68] demonstrated that the local abundance and pattern of distribution of some common lichen species on carbonate rock is related to patterns of propagule dispersal and also found that the spatial distribution of lichens on the stone-built surfaces is influenced by both species-specific patterns of propagule dispersal and microenvironmental requirements.

However, climatic and microclimatic conditions can only be controlled in indoor conditions and is almost impossible when the objects to be preserved are located outdoors, where other factors (such as architectural features) lead to some areas being more humid, which will probably result in faster recolonization. Faster recolonization (one month after on-site treatments) due to frequent rainfall was reported by Li et al. [49], who analysed the efficacy of four chemicals and two commercial biocides in inhibiting microbial colo-

Table 1
Studies dealing with recolonization of cultural heritage surfaces after cleaning treatment. Biocide = B [B^1: Biotin R; B^2: Biotin T; B^3: aqueous solution of Ca(ClO)$_2$; B^4: aqueous solution of NaDCC and CaCl2; B^5: H$_2$O$_2$; B^6: Octhilinone; B^7: AW-600; B^8: ROCIMA™ 103; B^9: Metatin N58-10/101; B^{10}: Bioestel; B^{11}: Cu nanoparticles; B^{12}: Ethanol; B^{13}: benzalkonium chloride; B^{14}: Preventol RI80; B^{15}: New Des 50; 6 mixture of quaternary ammonium compounds and octylisothiazolone; B^{17}: blend of biocides (oregano essential oil/2-methyl-4-chlorophenoxyacetic acid/methylene dithiocyanate/octhilinone)]; Mechanical Brushing = Mb; Water = W; Water Vapor = WV; Water repellent = WR; Consolidant = C; Protective coat = P; MW = Microwave.

Reference	Material/Organisms	Cleaning treatment and/or procedure	Recolonization related results
Bouichou et al. [9]	Concrete/Algae, lichen and moss	3 different B 2 different WV (Not specified)	*One year and a half* after treatment, signs of recolonization were observed on some of the testing areas. Recommendation: to combine water vapor cleaning with a preventive biocide product.
Coutinho et al. [66]	Tiles/Fungi	(TiO$_2$) Sol-Gel	The TiO2 coating after *forty days* did not prevent fungal growth and was not suitable for cultural heritage application.
de los Rios et al. [10]	Carbonate rocks/Lichen, moss, and cyanobacteria	B^1 + Mb with B^1 B^2 + Mb with B^2	After *two months* samples cleaned with Biotin T presented living fungal hyphae in fissures that promote lichen and/or fungal recolonization of the rock. In samples cleaned with Biotin R there were no fungal hyphae penetrating the stone or endolithic cyanobacteria.
Gabriele et al. [65]	Limestone/Cyanobacteria, green algae and diatoms	B^3 embedded in gel + P B^4 embedded in gel + P	After *two years* there were complete removal of biofilms and restoration of the original chromaticity.
Hallmann et al. [67]	Marble/Algae and fungi	Mb + WV + B^5+ C	*One year* after cleaning there were differences in fungal composition between the old grown biofilm and the treated surface, but no essential differences in the green algal community before and after cleaning could be observed.
Jurado et al. [11]	Lime mortar and sahcab/ Cyanobacteria, Bryophyta and Ascomycota	Mb + WR	Recolonization of microorganisms *1 year* after cleaning caused deterioration by increasing porosity of the mortar substratum. Samples collected after restoration works revealed a lower diversity than before the intervention.
Li et al. [49]	Limestone/Fungi and bacteria	B^6 B^7	Microorganisms recolonized the surface *5 and 10* days after octhilinone and AW-600 treatment respectively. Frequent rainfall might have been the reason for the recolonization.
Mascalchi et al. [18]	Marble and sandstone/ Lichen, black fungi, green algae and cyanobacteria	Mb + B^8+ Mb + WR Mb + MW + WR	The biocide was efficient in killing the biological growth; almost no recolonization was observed after *five years*. The microwave treatment was effective on biofilms and lichens, eliminating also cells in the bulk of the substrata, but recolonization was observed after 15 months.
Morando et al. [68]	Carbonate sedimentary rock/ Lichen	Not provided	Species with high potential for long range dispersal of ascospores may easily support recolonization dynamics after cleaning interventions. Species with low dispersal rate are less effective in rapid recolonization.
Nascimbene & Salvadori [69]	Calcareous rock/Lichen	B^9+ Mb + C + P	Effectiveness in the long term proved to be very low. Recolonization was evident *seven, eight and thirteen years* after treatment, exceeding 50% of the mean cover before restoration and involving four species-poor lichen communities
Nascimbene et al. [70]	Limestone/Lichen	B^9+ Mb + C + P	Recolonization by lichens covered c. 60% and 70% of the surface eight *and twelve years* after treatment. Total number of species was higher before restoration. The use of water repellents failed in avoiding lichen recolonization in the long term
Pinna et al. [71]	Marble, sandstone, and plaster/Lichen and fungi	Mb + B^{10} with C Mb + B^{11}+ WR Mb + B^{11}+ C Mb + B^{11}+ C	The *8-year*-long study showed that recolonization after treatments related mainly to substrate bioreceptivity and climatic conditions. Although treatments were effective in reducing recolonization, they did not play a crucial role in preventing biofilms and lichens growth.
Pozo-Antonio et al. [38]	Shale/Green algae and cyanobacteria	Mb + B^{12}+ Mb Mb + B^{13}+ Mb Mb + B^2+ Mb Mb + L (1064 nm) Mb + L (266 nm)	Unlike lasers, chemicals achieved overall satisfactory results after *4 years*. Cleaning effectiveness, harmfulness and durability of chemicals were highly influenced by the orientation of the schistosity planes of the stone.
Sanmartín et al. [19]	Granite/Algae	Mb W B^{12} B^{13} B^1 B^2 B^{14} B^{15}	Recolonization of the test areas was barely noticeable *one year* after treatments, Preventol RI80® proved to be the most effective, followed by benzalkonium chloride and the ethanol-water (1:1) mixture.
Sohrabi et al. [12]	Limestones/Lichen and cyanocacteria	Mb	High resilience of endolithic and nitrophytic communities to routinely (for 7 years) performed mechanical cleaning
Urzì et al. [72]	Limestone/Bacteria, cyanobacteria and algae	Mb + B^{16}	Treatment had little effect against cyanobacteria, while the bacterial population increased in numbers but changed drastically in terms of diversity. Some bacteria proliferate at the expense of the organic matter released by dead microorganisms.
Vannini et al. [51]	Basic and eutrophicated stone/Lichen	B^2 B^{14}	The substantial loss of vitality following treatments with Biotin T and Preventol persisted over time, and no physiological recovery was found after *90 days*.

(*continued on next page*)

Table 1 (*continued*)

Reference	Material/Organisms	Cleaning treatment and/or procedure	Recolonization related results
Zhu et al. [73]	Limestone and sandstone/ Algae, fungi, bacteria, lichen, and mosses	B^{17}+ WR + C	Biocide blend notably impacted the biological control of recolonization. Considerable difference between the microbial species compositions and community structures before and *one year* after biocide treatment.

nization on Feilaifeng Limestone cultural heritage. In a similar way, although micro and macroclimatic parameters were not systematically analysed in evaluating the efficacy of treatments, Mascalchi et al. [18] considered these parameters when analysing statues in different parks and indicate that the effects of these parameters may explain why recolonization occurs in some areas but not in others.

Biocolonization largely depends on substrate bioreceptivity (susceptibility to colonization by living organisms), together with climatic conditions. After the application of mixtures of consolidants, water-repellent products and biocides to trial areas of three substrates (marble, sandstone and plaster) in the Fiesole archaeological site (Firenze, Italy), Pinna et al. [71] showed that the recolonization of the three substrates 8 years after the treatment was mainly due to the bioreceptivity and the climatic conditions. Sohrabi et al. [12] examined the risk of biodeterioration processes associated with the presence of lichen on two types of limestone and also examined the presence of lichen 7 years after mechanical cleaning. These researchers showed that colonization and deterioration patterns were not unusual relative to those observed in studies of similar communities in different climatic regions and were generally associated with the different lithological characteristics.

Many studies have investigated the primary bioreceptivity of rocks present in heritage and/or commonly used in construction (e.g. Refs. [17,77–80]). The findings of these studies show that certain intrinsic properties of the rock significantly affect the growth of colonizing organisms on the surface; it has been widely demonstrated that high levels of surface roughness and of porosity accessible to water are the main characteristics favouring biological colonization [78,79]. Although numerous studies have addressed primary bioreceptivity (in unaltered material), the same is not true for either secondary bioreceptivity (affecting materials that have undergone some type of deterioration) or tertiary bioreceptivity (affecting materials that have received some type of treatment), although the modification of stone-built properties, either by weathering processes or as a result of artificial treatments, may be important. The bioreceptivity has been shown to increase when salts crystallize on the surface of granite as the surface roughness is affected [81]. These results seem indicate that the use of different treatments, such as those indicated above (brushing, biocides, laser, etc.), could affect some properties of the stone-built heritage, and could therefore increase both secondary and tertiary bioreceptivity and hence favour recolonization.

Regarding the changes in stone bioreceptivity due to application of treatments, Jurado et al. [11] concluded that, after cleaning and mechanical removal of biodeteriogens from the walls of San Roque church in Campeche (Mexico), restoration of the heavily colonized walls did not produce the expected result, and it even hastened the bioreceptivity of the material and biodeterioration processes. Sanmartín et al. [20] carried out an experiment with samples of shale colonized by lichens and cleaned with water, biocide, IR laser irradiation and UV laser irradiation and concluded that all treatments, except the biocide, increased the bioreceptivity of the shale, so that recolonization would be expected to occur more quickly. Coutinho et al. [66] tested the use of titanium dioxide (TiO_2) sol-gel coatings to prevent biological colonization on Portuguese glazed tiles; however, these researchers found that the TiO2 coating actually promoted fungal growth, by causing cracks to form on the surface of the glazed tiles, which increased the bioreceptivity of the surface and also led to aesthetic changes.

The length of time after treatment is another important factor in recolonization studies. It is not clear how long recolonization must be prevented for a removal treatment to be considered effective. The time considered in the different studies listed in Table 1 ranged from one month [49] to 25 years [68]. In all these studies, recolonization occurred within the considered time of each study, except in that by Gabriele et al. [65] on the effect of the application of an alginate-oxidizing biocide hydrogels followed by protective acrylic coatings on two UNESCO World Heritage Sites, in which no recolonization had occurred after two years.

The data in Table 1 indicate that recolonization by fungi was faster than recolonization by other organisms. However, it is not yet possible to explain this observation, because the studies address different aspects of recolonization. Thus, the effect may be due to a low treatment efficacy or to more favourable climatic conditions or to the materials being more bioreceptive, or all of these.

Regarding the composition of the recolonizer communities, fewer than half of the studies listed in Table 1 characterized the species present before and after the cleaning treatments. However, the composition of recolonizer communities appears to be less diverse than that of the communities present before treatment, and the organisms involved are generalists rather than specialists [11,69–73] with the recolonizers being different species than the previous colonizers. However, in most of the studies the monitoring was carried out in the medium term, before ecological succession was completed. Specialist species probably become established at later stages of the succession. Thus, in a study performed by Sohrabi et al. [12] on seven monumental buildings of the Pasargadae UNESCO-world heritage site (Iran) 7 years after a cleaning treatment, recolonization by the previous colonizing organisms was detected. However, Nascimbene et al. [70]and Nascimbene and Salvadori [69] only detected generalist recolonizer species even after 13 years treatment.

It is clear that biological removal treatments should be considered a first step in preventing biological colonization and biodeterioration of stone cultural heritage elements. Preventive measures are crucial to delaying recolonization after removal treatments, especially in stone-built cultural heritage exposed to the environment. The previous biological colonization favours subsequent rock-water relationships by increasing porosity and developing microfractures [82]. In such cases it should be considered to complement biological removal treatments by other control methods such as application of water repellents and/or consolidants. The use of water repellents after biological colonization removal treatments has been shown to delay recolonization by maintaining surface hydrophobicity [24]. Martinez et al. [83] evaluated antifouling effects of water repellent and photocatalytic treatments on mortars and observed that algal growth slowed significantly in cases of water run-off and even ceased altogether in cases of capillary rise of water. However, other studies have shown that the application of water repellents and consolidant products after biocidal treatments can cause aesthetically undesirable patterns such as streaking or surface cracks that can give rise to the formation of preferential water channels favouring biological recolonization [70,84,85].

In recent years, the application of water repellents or consolidants together with biocides has been investigated. Pinna et al. [86] applied Bioestel (mixture of a consolidant (tetraethylorthosilicate) and two biocides, tributyltin oxide (TBTO) + dibutyltin dilaurate) and Cu nanoparticles as biocide mixed with a consolidant (Estel 1000) or a water-repellent (Silo 111) to three stone substrates with different levels of bioreceptivity. Zhu et al. [73] applied a novel blend of biocides (oregano essential oil (OEO)/2-methyl-4-chlorophenoxyacetic acid (MCPA)/methylene dithiocyanate (MBT)/octhilinone (OIT)) mixed with a water repellent (acrylic acid composite) and a consolidant (Magnesium-Based Composite) to weathered and fresh sandstone blocks typically used in monuments in western and southern China. Both studies showed that combined treatments provide more effective long-term protection than a single-component biocides followed by application of water repellent and/or consolidant products as interference with the biocide layer was prevented.

4. Conclusions

Although many studies have investigated the efficacy of treatments used to eliminate biological colonization from cultural heritage, the treatment efficacy is only considered from the point of view of the level of cleaning achieved, and very few consider the possible subsequent recolonization. Moreover, it is difficult to draw general conclusions from the existing studies, as each involves different treatments, target organisms, substrates and climatic conditions (often not considered), and various methodologies are used. It is therefore not possible to make valid comparisons.

Regarding the substrate, most studies have been carried out on limestones, and the results cannot be fully extrapolated to other rocks with different physical properties (especially those related to water movement inside the rock) and with different levels of bioreceptivity. This lack of knowledge must be addressed in order to improve methods of cleaning stone-built heritage.

In general, most of the studies addressing recolonization involve calcareous cultural heritage after removal of lichens, cyanobacteria and algae using biocides. Recolonization of the substrate after the use of other removal methods or recolonization by other organisms as fungi and bacteria are less well studied. It seems that recolonization by fungi occurs faster than recolonization by other organisms; however, because of the above-mentioned impediments to comparing findings for different substrates and climatic conditions, it is not clear whether the faster recolonization is caused by the biocidal treatments or by other factors.

To consider the time factor is important in recolonization studies as time is one of the factors governing ecological succession. Thus, studies of species present shortly after treatment may detect only pioneer species, while long term surveys may reveal a more stable community that is probably more similar to the previous colonizer community.

Based on current knowledge and taking both biological removal and recolonization into account, we believe that application of biocides is the most effective treatment. However, because of the environmental problems related to their use, efforts should be led to find strategies to extend the long-term effects of biocides. In this respect, the use of water repellent or consolidant products after removal of biological colonization should be explored.

Further studies involving cleaning of stone-built cultural heritage should consider recolonization. With this aim, methodologies, treatments, organisms, primary, secondary and tertiary substrate bioreceptivity, and macro and microclimatic conditions should be considered before and during cleaning tasks and also at different times after the treatments.

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CRediT authorship contribution statement

B. Prieto: Conceptualization, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Project administration. **G. Paz-Bermúdez:** Conceptualization, Investigation, Project administration, Writing – original draft. **M.E. López de Silanes:** Conceptualization, Investigation, Validation, Writing – original draft. **C. Montojo:** Conceptualization, Investigation, Validation, Writing – original draft. **D. Pérez-Velón:** Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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