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Abstract

This work analyzed the influence of different application protocols on the efficacy of two biocides against the foliose lichen *Xanthoparmelia tinctina* on the sandstones of the Roman Archaeological site of Luni (Italy). The hypotheses that (a) biocide application tools (brush vs. poultice), (b) pre-treatment hydration, and (c) post-treatment washing may affect devitalization success were verified by monitoring chlorophyll *a* fluorescence of thalli, both *in situ* and in laboratory conditions. The hypothesis that (d) stone substrate may act as reservoir for later biocide release under repeated cycles of wetting and drying was also assayed. Analyses confirmed the importance of the application tool, with cellulose poultice being more effective than brush. Hydration influenced the biocide absorption by thalli. Moreover it modulated the metabolic activity and susceptibility to the available toxic compound, hindering lichens from entering a dormant state to tolerate stress. Depending on the preparation solvent (water vs. white spirit), the biocide application benefited from pre-treatment hydration and/or a post-treatment washing. Lastly, we showed that different sandstones variously adsorb the biocides and potentially contribute as a reservoir for their long-term release at low concentrations during successive hydration events.

Keywords

benzalkonium chloride, chlorophyll *a* fluorescence, lichen, thallus hydration, stone conservation

Highlights

The protocol to apply biocides on lichens affects the devitalization effects
Cellulose poultice application of biocides is more effective than that by brush
Pre-hydration and/or post-washing of thalli regulate biocide effectiveness
The stone substrate acts as a reservoir for long-term release of biocide
Effective application protocols can limit useless chemical release to the environment

1. Introduction

The growth of lithobiontic (micro-)organisms widely affects the aesthetic and threatens the durability of heritage surfaces (Caneva et al. 2008; Negi and Sarethy 2019). In particular, lichens are primary agents of stone biodeterioration. Their metabolites induce mineral leaching and biomineralization, and their hyphal penetration promotes disaggregation processes (Adamo and Violante 2000; Favero-Longo et al. 2005; Seaward 2015). Despite some bioprotective effects are recognized for certain species on certain lithologies (Salvadori and Casanova-Municchia 2016), the removal of lichens is generally considered pivotal to preserve heritage surfaces and is standard practice in conservation and restoration plans (Pinna 2017).

Physical methods for the control of lithobionts (e.g., electromagnetic wavelengths, laser and temperature shifts) have attracted recent research interests and showed promising results (e.g. Tretiach et al. 2012; Mascalchi et al. 2015; Sanz et al. 2015; Rivas et al. 2018). Nevertheless, their optimization and practical applicability at the scale of monumental surfaces is still pending (Pozo-Antonio et al. 2019; Sanmartín et al. 2019). Accordingly, interventions including devitalization of thalli by biocide application, followed by their removal by mechanical methods, are still commonly used by restorers (Kakakhel et al. 2019). Killing lichens prior to their brushing or scraping from the stone surfaces is recognized as a crucial need to prevent the persistence of viable thalline fragments within rock fissures and the dispersal of propagules, which may promote rapid recolonization processes (Pinna 2017). However, the effectiveness of biocidal treatments against lichens is not generalizable, and unsuccessful applications are widely documented in terms of poor devitalization results as well as of an undesired boosting of more resistant and aggressive species (Seaward 2015). It has been demonstrated that the effectiveness of biocidal products is species- and site-specific and it is strongly influenced by the application tools adopted (Favero-Longo et al. 2017). *In situ* preliminary assays are thus necessary to evaluate the site- and species-specific devitalization power of biocidal products and application tools, before their wide scale use in restoration interventions (Ascaso et al. 2002; de los Ríos et al. 2012; Favero-Longo et al. 2017; Pinna 2017). Certain practical steps of biocide application, which may affect their effectiveness, are similarly worthy of investigation to validate protocols ensuring the devitalization success.

Different substrate lithology and (micro)climatic conditions are site-related factors which may alter the effects of biocide applications (Caneva et al. 2008; Salvadori and Charola 2011). In strict relation to microenvironmental variation, the susceptibility of lichens to stress factors depends on their hydration state. They are stress-tolerant when dry, while highly sensitive when hydrated (even partially) and thus metabolically active (Tretiach et al. 2012). However, the choice of applying biocides on previously hydrated or dry thalli is still a controversial issue. Two contrasting hypotheses have been formulated, postulating that the pre-hydration of lichen thalli may assist the biocide absorption or, oppositely, that it may favour a quicker washing off and reduce absorption (Nugari and Salvadori 2003; Pinna 2017). Nevertheless, to the best of our knowledge, this issue has not yet approached experimentally . Similarly, it was hypothesized that the post-hydration may accelerate the action of the biocide (Tretiach et al.

2007). However, the practice of washing the treated surfaces some hours after biocide application to limit potential interferences with the stone substrate (Nugari and Salvadori 2003) was never evaluated in terms of treatment effectiveness.

In this work, we aimed to verify the primary hypothesis that (a) biocide application tools, (b) pre-treatment hydration step, and (c) post-treatment washing may, either singularly or in combination, affect the effectiveness against lichens of biocides having different active principles and dilution solvents. In particular, the effectiveness of different biocide treatments against a foliose lichen, performed both in an archaeological site and in laboratory conditions, was tested in terms of chlorophyll *a* fluorescence of the thalli with respect to a vitality threshold (F_V/F_M = 0.15; Favero-Longo et al. 2017). We also verified the additional hypothesis that (d) stone substrate may act as reservoir for later biocide release under repeated cycles of wetting and drying.

2. Materials and methods

2.1. Study site and lichen species

Biocide applications were performed, *in situ*, on the walls of the Amphitheatre of the Roman Archaeological site of Luni [Luni, La Spezia, Italy: UTM ED50, N 4879338, E 581882; 3 m]. Sandstones blocks of the Macigno Formation from Lunigiana were the main rock substrate. The Macigno Formation consists of fine to coarse sandstones with a variable degree of sorting that are mainly composed of quartz, feldspar and lithic grains (Franzini et al. 2007). Ripple cross-lamination locally occurs in fine grained samples. Carbonate cement is scarce and some clay may be present among grains.

Treatments were performed on the foliose lichen *Xanthoparmelia tinctina* (Maheu & A. Gillet) Hale, a species common from the submediterranean to the montane belt of Italy on siliceous rock surfaces, including the stone cultural heritage (Nimis et al. 1992). A total of 96 thalli were selected and treated *in situ* in April 2018 and May 2019. Lichen identification was performed in the field and checked in the laboratory following Giordani et al. (2002).

2.2. Biocide application in situ

Benzalkonium chloride (BAC) as 3% water solution of Preventol RI80 (alkyl dimethyl benzyl ammonium chloride, approx 80%, and isopropyl alcohol, 2%, in water; Lanxess, Köln, Germany), and N-octyl-isothiazolinone and 3-iodo-2-propynyl-N-butylcarbamate (OIT-IPBC) as 3% solution of BiotinR (OIT, 3-5%, and IPBC, 10-25%, in diethylene glycol butyl ether; CTS, Altavilla Vicentina, Italy) in white spirit (Kelix, Thormax Italia, Roma) were selected as biocides. They were applied either (i) using a paint-brush or (ii) with a cellulose poultice (Arbocel BC 1000, JR Pharma, Rosenberg, Germany), (i') after having moistened the thalli with sprayed water or (ii') avoiding this pre-hydration step. Per each surface unit of thallus, brush applications required approx. 0.3 mL cm^{-2} of diluted biocides; the applied poultice layer, approx. 1 cm thick, contained approx. 12 mL cm^{-3}. The cellulose poultice was covered with a cotton fabric for 4 h and later gently removed with a small spatula, thereafter (i'') washing the thalli or (ii'') avoiding this washing step. Thalli treated with water only in place of biocides were assayed as negative controls. Three thallus replicates per biocide per application method were examined [i.e. 3 replicates × (2 biocides + 1 control) × 2 application tools × 2 pre-treatment approaches × 2 post treatment approaches]. Treatments including the pre-hydration step were performed in April 2018, and the others in May 2019. Bottled water with low mineral content (Fonti di Vinadio, Vinadio, Italy) was used as control, and for the biocide dilution and the pre-hydration and washing steps.

Daily meteorological data (air temperature, relative humidity, rainfall) for the week prior and after the biocide applications in April 2018 and May 2019 were obtained from the nearby monitoring station of Luni (ARPA Liguria, 2018 and 2019; Fig. S1).

2.3. Biocide application in laboratory conditions

The application of BAC with the cellulose poultice was also tested in laboratory conditions. Treatment was performed on 14 thalli of *Xanthoparmelia* collected from a natural outcrop at Borgata Croux [Saint Cristophe, Aosta, Italy: UTM ED50, N 5068915, E 370323] together with their silicate (gneiss) substrate, avoiding any damage to Luni heritage surfaces. Biocide application was performed on thalli with and without pre-hydration step (moistening with sprayed water). Seven replicates were performed for each condition.

2.4. Lichen vitality measurements

Chlorophyll *a* fluorescence measurements (Chl_aF) - recognized as a tool for checking the vitality of photosynthetic organisms (Tretiach et al. 2008) - were carried out on *X. tinctina in situ* one day before (T0) and one day after (T1) biocide treatments, using a Handy-PEA fluorimeter (Plant Efficiency Analyser, Hansatech instruments Ltd., Norfolk, England), . Analyses were performed on dark-adapted thalli, covered overnight with a black cotton fabric, which were moistened by sprayed water just before the measurements, to avoid that the additional hydration may further affect the biocide action. Measurements on the thalli treated in the laboratory were carried out one day before the biocide application (T0), immediately after the removal of cellulose poultice (T4h) and one day after (T1). Analyses were performed following the protocol adopted *in situ*, with the exception that the moistening at T0 was avoided for thalli foreseen without the pre-hydration step, and that measurements at T1 were performed for all thalli both before and after their moistening.

Five measurements were taken on each thallus, positioning the sensor head at 90° over its surface, inducing Chl_aF by a red light (peak at 650 nm), and recording the data after a saturating light pulse of 1s (Malaspina et al. 2014). Chl_aF increases from F_0, when all the reaction centres of PSII are open, to F_M, when all the reaction centres of PSII are closed. The maximum quantum efficiency of PSII, that is F_V/F_M (where F_V=F_M-F_0), a temperature-independent parameter of Chl_aF emission, and variations in F_0, related to chlorophyll contents of the light harvesting complex (Baruffo and Tretiach 2007), were used to check the vitality of the thalli, in agreement with previous researches on the effectiveness of biocidal treatments against lichens(e.g.Tretiach et al. 2012; Favero-Longo et al. 2017).

2.5. Biocide absorption by lichen thalli

At the end of the fluorescence measurements at T1, the *X. tinctina* thalli treated *in situ* with BAC without performing the pre-hydration step, and the overall set of thalli treated in the laboratory, were gently detached from the rock substrate with a scalpel and processed to analyse the absorbed BAC. In particular, they were carefully cleaned under a stereomicroscope and then left overnight in a climatic chamber at 16°C and 55% of relative humidity (residual water content <10%). Samples of 50 mg were homogenized with 1 mL of deionized water and centrifuged at 20,000 rfc for 10 min. The supernatant was filtered at 0.45 µm using a syringe filter and 30 µL of the solution were directly analyzed by HPLC (Water LC I Plus). BAC was separated using a Phenomenex C18 (250 x 4.6 mm, particle size 5 µm) using a mixture of acetonitrile-sodium acetate buffer (pH 5.0; 0.2 M) (70:30, v/v) as mobile phase with flow rate 1 mL/min (Rojsitthisak et al. 2005). Runs were monitored at 210nm. Quantification was performed with a calibration curve (5 – 50 µg/mL) of BAC from Sigma-Aldrich (≥ 95.0%). The limit of quantification of the analysis was 0.04 µg mg^{-1}.

2.6. Adsorption and desorption of benzalkonium chloride by sandstone lithologies

The property of different sandstone lithologies to adsorb and desorb BAC upon its application on the rock surface and a subsequent washing with deionized water was assessed in the laboratory. In particular, four sandstone blocks of the Macigno Formation, similar to those used in the Amphiteatre of Luni, were collected on the banks of the Parmignola, a stream located at few hundreds of meters from the Roman site, and cross sectioned with a diamond saw (section thickness >5 cm). In the central parts of the cut surfaces, parcels (2×3 cm) were established and treated with 250 µL of 3% BAC (Sigma-Aldrich, St.Louis, MO, USA), applied with a Transferpipette 100-1000 µL (Brand, Wertheim, Germany). The parcels were let to dry overnight at room temperature. Thereafter, 250 µL of deionized water were applied on each parcel and (after 30 seconds) a double layer of absorbent paper (9 mg cm^{-2}) was applied -to simulate the potential absorption of a lichen thallus- and let dry on the rock surface. The absorbent paper was then suspended in 2 mL of deionized water to extract BAC, which was quantified as described above. Blocks of other sandstone lithologies employed in the Italian stone cultural heritage were also cross-sectioned and similarly processed for comparison, including the Pietra Serena, widely used in Tuscany (Fratini et al. 2014), the Cortemilia sandstone, from Southern Piedmont (Gelati et al. 2010), and the sandstone of the Verrucano Lombardo Formation, well known for rock-art in the Valle Camonica (Brack et al. 2008). At least three parcels per treatment (BAC, water) were considered per each block. Moreover, the same process was repeated on glass slides, as negative control.

Thin cross sections prepared from the rock blocks were observed by plane polarized light microscopy to characterize their mineral composition and texture. Scanning electron microscopy in back scattered electron mode (SEM-BSE), undertaken with a JEOL JSM IT300LV (High Vacuum - Low Vacuum 10/650 Pa - 0.3–30 kV) and coupled with image analysis by the software WinCAM (Regent's Instrument, Canada), was used to estimate total porosity (Favero-Longo et al. 2009).

2.7. Statistics

Generalized Linear Models (GLMs) were applied to describe the effects of the different devitalization protocols on photobiont vitality *in situ* at T1, with the applied products (BAC, OIT-IPBC, and water as control), the application tools (brush and cellulose poultice), the pre-hydration and washing steps being considered as independent predictors. In particular, a factorial ANOVA analysis was performed to detect significant differences in F_V/F_M and F_0 according to the different predictors (product, application tool, prehydration, washing). GLM analyses were carried out with SYSTAT 10.2 (Systat Software Inc., San Jose, CA).

For all the analyses *in situ* and in the laboratory, significant differences in F_V/F_M at T1 between the different study cases and, for each study case, with respect to a viability threshold (set at F_V/F_M = 0.15, see Favero-Longo et al. 2017, with refs. therein) were analyzed by means of ANOVA with post-hoc Tukey's and t-test, respectively, using SYSTAT 10.2 ($P<0.05$ as significant). For each study case, significant differences of F_0 in the thalli treated with biocides with respect to the control ones were assessed at T1. Significant differences in the absorption of BAC by lichen thalli, and in the BAC desorption patterns by different sandstone lithologies, were also examined by means of ANOVA with Tukey's post-hoc test.

3. Results

3.1. Efficacy of devitalization treatments in situ

GLM analyses (Table 1) showed that all the considered factors (product, application tool, pre-hydration and washing) contribute to determine the efficacy of devitalization treatments, evaluated in terms of F_V/F_M and F_0 of the targeted *Xanthoparmelia* thalli.

F_V/F_M values of thalli treated with biocides, independently of the application tool and the hydration protocol, were significantly lower than controls (Fig. 1). However, only in some cases values decreased below the viability threshold (F_V/F_M = 0.15; Favero-Longo et al., 2017, with refs. therein). In particular, biocide application by brush was effective for OIT-IPBC, but only when coupled with thallus pre-hydration and/or post-treatment washing. Application with cellulose poultice was generally effective for BAC, while the effectiveness of OIT-IPBC was lower when thalli were not washed.
F_0 values (Figs. S2-S3) strongly decreased with respect to controls only for OIT-IPBC application on pre-hydrated thalli (mean±SE: -62±8%), in particular when thalli were not

washed (-71±9%). A relative increase of F_0 (144±13%) followed all the applications of OIT-IPBC on non pre-hydrated thalli. BAC induced only slight decreases of F_0 with respect to controls (-16±3%).

3.2. Efficacy of devitalization treatments in the laboratory

In the laboratory, the application of BAC with cellulose poultice was effective against thalli moistened before the treatment, while F_V/F_M of non pre-hydrated thalli did not significantly decrease beneath the vitality threshold of 0.15 (Fig. 2). In particular, fluorimetric measurements before the biocide application (T0) confirmed the well-known difference between the F_V/F_M of moistened thalli (ca. 0.7) and dry thalli (ca. 0.07). At the removal of the cellulose poultice (T4h), without any additional moistening, F_V/F_M of thalli treated in the wet state was significantly lower than the vitality threshold and with respect to thalli treated in the dry state. At T1, all thalli were dehydrated and F_V/F_M was significantly below 0.15, but after their moistening, those which had received the poultice application in the dry state recovered F_V/F_M values significantly higher than the threshold.

3.3. Biocide content in lichen thalli

The absorption of BAC was detected and quantified in all lichen thalli treated *in situ* without performing pre-hydration, and in those treated in the laboratory, but concentrations strongly differed depending on the application method (Fig. 3).

In situ, the thalli treated with cellulose poultice and not exposed to the final washing step displayed one order magnitude higher content of BAC (mean 1.4 µg mg^{-1}) with respect to those washed after the poultice removal and those treated with brush (0.1 µg mg^{-1}). In these latter, the BAC content was similarly low, irrespective whether the final washing was performed or not.

In the laboratory, the content of BAC absorbed by thalli which were moistened before the application with cellulose poultice and not washed (mean 1.8 µg mg^{-1}) was similar to that detected *in situ* with the same application tool, but without pre-hydration. By contrast, the biocide content of thalli treated with cellulose poultice in the dehydrated state was significantly lower (0.2 µg mg^{-1}).

3.4. Adsorption and desorption of benzalkonium chloride applied on sandstones

The amount of BAC desorbed from the rocks upon a re-wetting cycle, and thus absorbable by the absorbent paper used as to simulate the lichen thallus, was extremely low (always <0.5%; Fig. 4). The sandstone of the Verrucano Lombardo Formation showed a significantly higher desorption (0.34%), but remarkable differences were also detectable between the blocks of the Macigno sandstone, with values ranging from 0.15% (L2) to the detection limit(<0.03%; L1, L3, L4). The recovery of BAC from a glass slide (non-adsorbing substrate) was two order of magnitude higher, above 30%.

On the basis of SEM-BSE observations (Fig. S4), the Verrucano Lombardo showed an intrinsic porosity remarkably lower than that of Macigno sandstone. Accordingly, BAC barely entered the rock volume and, upon the drying step, recrystallized directly on the surface, from which it was mobilized during the subsequent re-wetting. Oppositely, in the case of the other sandstones, the applied biocide clearly entered the rock volume. In the case of the Macigno sandstones, microscopic observations of petrographic thin cross sections showed that a clay fraction occurred in L1, L3 and L4, while it was absent in L2 (Fig. S5). Pietra Serena showed a fitted fabric due to pressure dissolution, with juxtaposed grains and absence of cement or matrix, while the Cortemilia sandstone showed traces of carbonate cement and a clay fraction. A fine-grained sericitic matrix possibly characterized the block of Verrucano Lombardo.

4. Discussion

Our findings support the hypothesis that the protocol adopted to apply biocides significantly affects the devitalization of lichen thalli (Fig. 5). Besides confirming the importance of the application tool, with cellulose poultice being more effective than brush (Favero-Longo et al. 2017; Matteucci et al. 2019), this experimental work clarified the remarkable influence of the state of hydration of lichen thalli on their susceptibility to biocides. Hydration modulates the biocide absorption by thalli. Moreover, it controls their maintaining an active metabolism or entering a dormancy state, thus succumbing to or tolerating, respectively, the available toxic compounds. In relationship with the water or organic solvent preparation of the assayed biocides, we highlighted the biocide-specific advantage of pre-treatment hydration and/or post-treatment washing of thalli to improve the application protocol effectiveness. In particular, the poultice application was necessary to make effective against *X. tinctina* the assayed water-solution of benzalkonium chloride (BAC), independently of the pre- or post-treatment hydration of thalli. Differently, the washing of thalli after the biocide application was necessary to make effective the assayed organic-solvent solution of N-octyl-isothiazolinone and 3-iodo-2-propynyl-N-butylcarbamate (OIT-IPBC), either applied by brush or with cellulose poultice. In this regard, until innovative strategies to control biodeteriogens will be routinely available, the conventional use of traditional biocides by restorers cannot overlook this necessity of adopting effective application protocols and hence limit the useless release of biocides in the environment. In addition, this work showed that the stone substrate, depending on the lithology, may variously absorb the applied biocide, potentially contributing as a reservoir for its long-term release at low concentrations during successive hydration events.

4.1. Biocide efficacy and thallus hydration

Lichen tolerance of extreme stress conditions is well documented and has been related to their ability to cyclically enter and leave a dormancy state by thallus dehydration and rehydration, respectively (Beckett et al. 2008). Such adaptation is supported by enzymatic and nonezymatic mechanisms to protect the integrity of cellular components and limit pro-oxidative processes (Kranner et al. 2008), an effective machinery to maintain proteostasis (Armaleo et al. 2019) and the interplay of the whole lichen microbiota (Cernava et al. 2019). A notable example is the tolerance to high temperatures, which for dry thalli ranges from 70°C to more than 100°C depending on species (Lange 1953), while it is generally lower than 45-50°C when thalli are forcedly maintained in the hydrated state (McFerlane and Kershaw 1978; Tretiach et al. 2012). In agreement, lichen resistance to gaseous pollutants, as SO_2 and O_3, is higher during the dry state; by contrast, the pollutants can dissolve in the hydrated thallus, in which the symbionts are metabolically active and sensitive to their toxic effects (Vannini et al. 2020).

A similar pattern is here confirmed for the foliose lichen *X. tinctina* treated with the water soluble BAC and OIT-IPCB prepared in white spirit. The quaternary ammonium salt BAC perturbs the phospholipid bilayer of the biological membranes, causing their damage and the cell lysis (Wessels and Ingmer 2013). OIT oxidazes thiol-containing cytoplasmic and membrane-bound compounds, yielding metabolic inhibition (Denyer and Stewart 1998), and IPBC disrupts the formation of fungal cell walls by interfering with synthesis of phospholipids and fatty acids (Biehl 2019). Despite their different active principles, target molecules and solubility, thallus hydration influences the effectiveness of both products.

The poultice application of BAC *in situ*, which always decreased F_V/F_M below the viability threshold set at 0.15, carried the water-dissolved biocide as well as contributed to maintain wet the pre-hydrated thalli and to hydrate the thalli in the dry state (Favero-Longo et al. 2017). Such latter effect was clearly recognizable in the laboratory assays, in which the very low F_V/F_M of the initially dry thalli remarkably increased at the time of the poultice removal (T4h), indicating its metabolic activation by water rather than its devitalization by the biocide. The dry state at the time of the application also implied in the laboratory a significantly lower BAC absorption with respect to that detected in the pre-hydrated thalli. Such findings agree with the linear positive correlation between the hydration of thalli and their efficiency to accumulate persistent organic pollutants (Kylin and Bouwman 2012; Augusto et al. 2012) and reject the hypothesis that pre-hydration may reduce the absorption of biocides (Tretiach et al. 2007).

In situ, BAC absorption in non pre-hydrated thalli was instead more similar to that of the pre-hydrated thalli in the laboratory. With this regard, it is worth noting that F_V/F_M of lichen thalli, and thus their metabolic activity, is also highly related to weather conditions during the two days prior to the measurements (Vivas et al. 2017). Differing from the thalli kept in the laboratory in the dry state, those in the study site were regularly exposed to high humidity levels during the night (RH above 80-90% in the days before the treatment; Fig. S1). Although they were dried at the time of biocide application (around noon), they likely had a higher attitude, in terms of physiological state, to recover their metabolic activity by effectively absorbing the water solution of BAC, even without the pre-treatment hydration step.

The application of BAC by brush contributed a lower quantity of biocide and did not maintain the hydration of the thalli, justifying a lower absorption and the poor devitalization effectiveness. The fact that post-treatment washing did not lead to a further lowering of F_V/F_M suggests that the effect was likely more limited by the biocide quantity than by a scarce metabolic activation. This also agrees with the fact that the absorbed BAC did not decrease with the post-washing step, suggesting that the available biocide had been effectively absorbed and retained by the cell structures. In this sense, neither brush nor poultice application determined at T1 a remarkable decrease of F_0, detectable upon the loss of chlorophyll following membrane integrity impairment (Vannini et al. 2018), suggesting that BAC-driven cell lysis had still not deeply proceeded and that some absorbed BAC could not be washed away.

In the case of OIT-IPBC, no difference was detected in the effectiveness of the assayed application tools, indicating that the lower quantity of active principles carried by the brush was sufficient to kill the lichens. However, for this biocide prepared in white spirit, the wetting of thalli by the pre-treatment hydration and/or the post-treatment washing was a necessary requirement to make the treatment effective. Accordingly, the removal of crustose and foliose lichens following the application of BiotinR with a protocol which does not mention hydration steps determined the persistance of thallus remains with some (few) viable photobiont cells (de los Ríos et al. 2012). As hypothesized for other biocides, but not experimentally verified (Tretiach et al. 2007), the post-treatment washing of thalli treated when dry showed the highest effectiveness in the F_V/F_M decreasing, suggesting that the lichen recovery of the metabolic activity in the presence of the toxic molecules was the most suitable method to favour its susceptibility and face its defence strategies. This agrees with the report that IPBC is highly soluble in organic solvents and poorly soluble in water (156 mg L^{-1} at 20°C; Juergensen et al. 2000), but its efficacy depends on the water dissolved fraction and its general wide use is related to strategies to allow its dissolution, including the predissolution in organic solvents (Steinberg 2002). However, the highest decrease of F_0 (>60%) was observed in thalli pre-hydrated, either washed or unwashed, suggesting that they mostly faced a strong damage of cell structures and the damage and loss of chlorophyll. In thalli treated when dry, instead, F_0 showed a relative increase, which may reflect the initial presence of some free chlorophyll due to membrane damage (Strasser 1997), or a resistance attempt towards a treatment with incomplete killing efficacy (Favero-Longo et al. 2017). Further investigations will be necessary to clarify such response patterns of thalli as well as to unveil if and how different application protocols may also variously impact the hyphal penetration component of lichens (*sensu* Favero-Longo et al. 2005) and the associated microbial communities, which already revealed different sensitivity to different biocidal products (de los Ríos et al. 2012) and play a crucial role in biodeterioration processes (Speranza et al. 2012).

4.2. Does substrate porosity influence the biocide efficacy?

The different effectiveness of biocides against the growth of algae inoculated on sandstone lithologies was related to their different porosity and clay contents (Young et al. 1995).

Biocides can penetrate below the surface and either be bio-available while bound to the minerals or be slowly desorbed and become available to (micro-)organism absorption under repeated cycles of wetting and drying (Cameron et al. 1997). For some biocides, the adsorption to clay minerals may determine their inactivation, but quaternary ammonium salts should maintain their biocidal activity when bound (Walters et al. 1973; Cameron et al. 1997). These processes, however, have been infrequently investigated (Koestler and Salvadori 1996) and their consequences for practical issues of restoration protocols -such as their effect on recolonization dynamics- are scarcely taken into account. In particular, they should be carefully considered with respect to the widespread application of biocides as a preventive tool to protect heritage surfaces from recolonization process, which is to maintain rock cleaning after the removal of lichens and biofilms (Pinna 2017).

In agreement with previous works (Young et al. 1995; Cameron et al. 1997), our laboratory experiment showed that the amount of BAC desorbed by a wetting event, following the biocide application and consequent rock adsorption, depends on physical and mineralogical properties of the different sandstone lithologies. In particular, the higher the rock porosity and the presence of clay minerals, the lower the biocide desorption at new rain events or watering. In the case of lithologies with very low porosity, such as Verrucano Lombardo, the biocide visibly crystallized at the rock surface and could likely be washed off by flowing water rather than persist as long-term protection (Cameron et al. 1997). Even within the same lithology, clay contents can vary from a block to another, as in the case of the Macigno sandstone in Luni, and thus differently affect biocide adsorption and desorption. For all the examined lithologies, the amount of desorbed biocide potentially available to microbial absorption is 2-3 orders of magnitude lower than that provided during the application, which turned effective only in the case of the copious poultice treatment. Accordingly, the biocides applied after the cleaning interventions may possibly exert their preventive protection insofar they remain abundantly bound within the rock porosity (Cameron et al. 1997), although the bio-activity should be demonstrated for each considered quaternary ammonium compound. On the other hand, such application strategy produces a reservoir for their gradual release at low and likely ineffective concentrations. With this regard, the phenomenon may be likely related to the reported cases of surface eutrophication following the application of quaternary ammonium salts, their degradation and consequent nitrogen supply, favouring recolonization processes by nitrophilous, fast growing species (Scheerer et al. 2009). Moreover, the release of low and ineffective concentrations of BAC can promote bacterial adaptation and antibiotic resistance (Kampf 2018; Kim et al. 2018; Poursat et al. 2019).

5. Conclusions

This work confirmed the hypothesis of a biocide-specific importance of the application tools, the pre-treatment hydration and/or the post-treatment washing to make the devitalization treatments effective against lichens (F_V/F_M of lichen thalli after the treatment <0.15).

Acknowledgements

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References

Adamo, P., Violante, P., 2000. Weathering of rocks and neogenesis of minerals associated with lichen activity. Appl Clay Sci 16, 229-256.

Armaleo, D., Müller, O., Lutzoni, F., Andrésson, Ó. S., Blanc, G., Bode, H. B., et al., Joneson, S., 2019. The lichen symbiosis re-viewed through the genomes of *Cladonia grayi* and its algal partner *Asterochloris glomerata*. BMC genomics 20, 605.

Ascaso, C., Wierzchos, J., Souza-Egipsy, V., De los Ríos, A., Rodrigues, J.D., 2002. In situ evaluation of the biodeteriorating action of microorganisms and the effects of biocides on carbonate rock of the Jeronimos Monastery (Lisbon). Int Biodeterior Biodegrad 49, 1-12.

Augusto, S., Máguas, C., Branquinho, C., 2013. Guidelines for biomonitoring persistent organic pollutants (POPs), using lichens and aquatic mosses–a review. Environ Poll 180, 330-338.

Baruffo, L., Tretiach, M., 2007. Seasonal variations of F_0, F_M, and F_V/F_M in an epiphytic population of the lichen *Punctelia subrudecta* (Nyl.) Krog. Lichenologist 39, 555-565.

Beckett, R.P., Kranner, I., Minibayeva, F.V., 2008. The stress physiology of the symbiosis. In: Nash III T.H. (Ed.), Lichen biology. Cambridge University Press, Cambridge, 134-151.

Biehl, Z.U., 2019. Review of the scientific and technological literature of fungicides in tannery industry: reducing the use and increasing the efficiency of fungicides in the leather industry. Journal of AQEIC 70, 33-42

Brack, P., Dal Piaz, G. V., Baroni, C., Carton, A., Nardin, M., Pellegrini, G. B., Pennacchioni, G., 2008. Note illustrative della Carta Geologica d'Italia alla scala 1: 50.000. Foglio 058, Monte Adamello. Carta Geologica d'Italia alla scala 1: 50.000. ISPRA, Roma.

Cameron, S., Urquehart, D., Wakefield, R., Young, M., 1997. Biological growths on sandstone buildings. Control and treatment. Technical advice note, 10. Historic Scotland (Technical Conservation, Research Education Division), Edimburgh.

Caneva, G., Nugari, M.P., Salvadori, O., (Eds.), 2008. Plant biology for cultural heritage: biodeterioration and conservation. Getty Publications, Los Angeles.

Cernava, T., Aschenbrenner, I. A., Soh, J., Sensen, C. W., Grube, M., Berg, G., 2019. Plasticity of a holobiont: desiccation induces fasting-like metabolism within the lichen microbiota. ISME J 13, 547-556.

de los Ríos, A., Pérez-Ortega, S., Wierzchos, J., Ascaso, C., 2012. Differential effects of biocide treatments on saxicolous communities: Case study of the Segovia cathedral cloister (Spain). International Biodeterioration & Biodegradation 67, 64-72.

Denyer, S. P., Stewart, G.S.A.B., 1998. Mechanisms of action of disinfectants. International Biodeterioration & Biodegradation 41, 261-268.

Favero-Longo, S.E., Castelli, D., Salvadori, O., Belluso, E., Piervittori, R., 2005. Pedogenetic action of the lichens *Lecidea atrobrunnea, Rhizocarpon geographicum* gr. and *Sporastatia testudinea* on serpentinized ultramafic rocks in an alpine environment. Int Biodeterior Biodegrad 56, 17-27.

Favero-Longo, S.E., Borghi, A., Tretiach, M., Piervittori, R., 2009. In vitro receptivity of carbonate rocks to endolithic lichen-forming aposymbionts. Mycol Res 113, 1216-1227.

Favero-Longo, S.E., Benesperi, R., Bertuzzi, S., Bianchi, E., Buffa, G., Giordani, P., Loppi, S., Malaspina, P., Matteucci, E., Paoli, L., Ravera, S., Roccardi, A., Segimiro, A., Vannini, A., 2017. Species-and site-specific efficacy of commercial biocides and application solvents against lichens. Int Biodeterior Biodegrad 123, 127-137.

Fratini, F., Pecchioni, E., Cantisani, E., Rescic, S., Vettori, S., 2014. Pietra Serena: the stone of the Renaissance. In: Pereira, D., Marker, B.R., Kramar, S., Cooper, B.J., Schouenborg, B.E. (Eds.), Global heritage stone: Towards international recognition of Building and ornamental stone. Geological Society, London, Special Publications, 407: 173-186.

Franzini, M., Leoni, L., Lezzerini, M., Cardelli, R., 2007. Relationships between mineralogical composition, water absorption and hydric dilatation in the "Macigno" sandstones from Lunigiana (Massa, Tuscany). Eur J Min 19, 113-123.

Gelati, R., Gnaccolini, M., Polino, R., Mosca, P., Piana, F., Fioraso, G., 2010. Note illustrative della Carta Geologica d'Italia alla scala 1:50000, Foglio 211 Dego. ISPRA-Istituto Superiore per la Protezione e la Ricerca Ambientale, pp. 117.

Giordani, P., Nicora, P., Rellini, I., Brunialti, G., Elix, J. A. (2002). The lichen genus *Xanthoparmelia* (Ascomycotina, Parmeliaceae) in Italy. Lichenologist, 34, 189-198.

Juergensen, L., Busnarda J., Caux, P.-Y., Kent, R., 2000. Fate, behavior, and aquatic toxicity of the fungicide IPBC in the Canadian environment. Environ Toxicol 15, 201-213.

Kakakhel, M.A., Wu, F., Gu, J.D., Feng, H., Shah, K., Wang, W., 2019. Controlling biodeterioration of cultural heritage objects with biocides: A review. Int Biodeterior Biodegrad 143, 104721.

Kampf, G., 2018. Adaptive microbial response to low-level benzalkonium chloride exposure. J of Hosp Infect 100, e1-e22.

Kim, M., Weigand, M.R., Oh, S., Hatt, J.K., Krishnan, R., Tezel, U., Pavlostathis, S.G., Konstantinidis, K.T., 2018. Widely used benzalkonium chloride disinfectants can promote antibiotic resistance. Appl Environ Microbiol 84, e01201-18

Koestler, R.J., Salvadori, O., 1996. Methods of evaluating biocides for the conservation of porous building materials. Science and Technology for Cultural Heritage 5, 63-68.

Kranner, I., Beckett, R., Hochman, A., Nash III, T.H., 2008. Desiccation-tolerance in lichens: a review. Bryologist 576-593.

Kylin, H., Bouwman, H., 2012. Hydration state of the moss *Hylocomium* splendens and the lichen *Cladina stellaris* governs uptake and revolatilization of airborne α-and γ-hexachlorocyclohexane. Environ Sci Technol 46, 10982-10989.

Lange, O.L., 1953. Hitze-und Trockenresistenz der Flechten in Beziehung zu ihrer Verbreitung. Flora oder Allgemeine Botanische Zeitung 140, 39-97.

Malaspina, P., Giordani, P., Faimali, M., Garaventa, F., Modenesi, P., 2014. Assessing photosynthetic biomarkers in lichen transplants exposed under different light regimes. Ecological Indicators 43, 126-131.

Mascalchi, M., Osticioli, I., Riminesi, C., Cuzman, O.A., Salvadori, B., Siano, S., 2015. Preliminary investigation of combined laser and microwave treatment for stone biodeterioration. Stud Conserv 60(Supplement 1), 19–26.

Matteucci, E., Scarcella, A.V., Croveri, P., Marengo, A., Borghi, A., Benelli, C., Hamdan, O., Favero-Longo, S.E., 2019. Lichens and other lithobionts on the carbonate rock surfaces of the heritage site of the tomb of Lazarus (Palestinian territories): diversity, biodeterioration, and control issues in a semi-arid environment. Ann Microbiol 69, 1033-1046

MacFarlane, J.D., Kershaw, K.A., 1978. Thermal sensitivity in lichens. Science 201, 739-741.

Negi, A., Sarethy, I.P., 2019. Microbial biodeterioration of Cultural Heritage: Events, colonization, and analyses. Microb Ecol 78, 1014-1029.

Nimis, P.L., Pinna, D., Salvadori, O. 1992. Licheni e conservazione dei monumenti. CLUEB, Bologna.

Nugari, M.P., Salvadori, O., 2003. Biocides and treatment of stone: limitations and future prospects. In: Koestler, R.J., Koestler, V.H., Charola, A.E., Nieto-Fernandez, F.E., (Eds.), Art, Biology, and Conservation: Biodeterioration of Works of Art. The Metropolitan Museum of Art, New York, pp. 518-535.

Pinna, D., 2017. Coping with biological growth on stone heritage objects: methods, products, applications, and perspectives. Apple Academic Press, Oakville

Poursat, B.A., van Spanning, R.J., de Voogt, P., Parsons, J.R., 2019. Implications of microbial adaptation for the assessment of environmental persistence of chemicals. Crit Rev Environ Sci Technol 49, 2220-2255.

Pozo-Antonio, J.S., Barreiro, P., González, P., Paz-Bermúdez, G., 2019. Nd: YAG and Er: YAG laser cleaning to remove *Circinaria hoffmanniana* (Lichenes, Ascomycota) from schist located in the Côa Valley Archaeological Park. Int Biodeterior Biodegrad 144, 104748

Rivas, T., Pozo-Antonio, J.S., de Silanes, M. L., Ramil, A., López, A.J., 2018. Laser versus scalpel cleaning of crustose lichens on granite. Appl Surf Sci 440, 467-476.

Rojsitthisak, P., Wichitnithad, W., Pipitharome, O., Sanphanya, K., Thanawattanawanich, P., 2005. Simple HPLC determination of benzalkonium chloride in ophthalmic formulations containing antazoline and tetrahydrozoline. PDA J Pharm Sci Technol 59, 332-337.

Salvadori, O., Casanova-Municchia, A., 2016. The role of fungi and lichens in the biodeterioration of stone monuments. Open Conference Proceedings Journal 7, suppl. 1 M4, 39-54.

Salvadori, O., Charola, A.E., 2011. Methods to prevent biocolonization and recolonization: an overview of current research for architectural and archaeological heritage. In: Charola A.E., McNamara, C., Koestler, R.J. (Eds), Biocolonization of stone: Control and preventive methods. Proceedings from the MCI Workshop Series. Smithsonian Contributions to Museum Conservation (Vol 2). Smithsonian Inst. Press, Washington, 37-50.

Sanmartín, P., Fuentes, E., Montojo, C., Barreiro, P., Paz-Bermúdez, G., Prieto, B. (2019). Tertiary bioreceptivity of schists from prehistoric rock art sites in the Côa Valley (Portugal) and Siega Verde (Spain) archaeological parks: Effects of cleaning treatments. Int Biodeterior Biodegrad 142, 151-159.

Sanz, M., Oujja, M., Ascaso, C., de los Ríos, A., Pérez-Ortega, S., Souza-Egipsy, V., Wierzchos, J.; Speranza, M., Vega Cañamares, M., Castillejo, M., 2015. Infrared and ultraviolet laser removal of crustose lichens on dolomite heritage stone. Appl Surf Sci 346, 248–255.

Scheerer, S., Ortega-Morales, O., Gaylarde, C., 2009. Microbial deterioration of stone monuments-an updated overview. Advances in Applied Microbiology 66, 97-139.

Seaward, M.R.D., 2015. Lichens as agents of Biodeterioration. In Recent Advances in Lichenology. In: Upreti, D.K., Divakar, P.K., Shukla, V., Bajpai, R. (Eds.), Recent advances in lichenology. Modern methods and approaches in biomonitoring and bioprospection, volume 1. Springer India, New Delhi, 189-211.

Speranza, M., Wierzchos, J., De Los Rios, A., Perez-Ortega, S., Souza-Egipsy, V., Ascaso, C., 2012. Towards a more realistic picture of in situ biocide actions: Combining physiological and microscopy techniques. Sci Total Environ 439, 114-122.

Steinberg, D.C., 2002. Iodopropynyl butylcarbamate as a preservative. Dermatitis 13, 207-208.

Strasser, B. J., 1997. Donor side capacity of photosystem II probed by chlorophyll a fluorescence transients. Photosynth Res 52, 147-155.

Tretiach, M., Crisafulli, P., Imai, N., Kashiwadani, H., Moon, K.H., Wada, H., Salvadori, O., 2007. Efficacy of a biocide tested on selected lichens and its effects on their substrata. Int Biodeterior Biodegrad 59, 44-54.

Tretiach, M., Bertuzzi, S., Salvadori, O., 2008. In situ vitality monitoring of photosynthetic organisms by chlorophyll a fluorescence techniques. In:Tiano, P., Pardini, C. (Eds.), In situ monitoring of monumental surfaces. Edifir, Firenze, pp. 279-286.

Tretiach, M., Bertuzzi, S., Candotto Carniel, F. 2012. Heat shock treatments: a new safe approach against lichen growth on outdoor stone surfaces. Environmental Science & Technology 46, 6851-6859.

Vannini, A., Contardo, T., Paoli, L., Scattoni, M., Favero-Longo, S.E., Loppi, S. (2018). Application of commercial biocides to lichens: Does a physiological recovery occur over time? Int Biodeterior Biodegrad 129, 189-194.

Vannini, A., Canali, G., Pica, M., Nali, C., Loppi, S., 2020. The water content drives the susceptibility of the lichen *Evernia prunastri* and the moss *Brachythecium* sp. to high ozone concentrations. Biology 9, 90.

Vivas, M., Pérez-Ortega, S., Pintado, A., Sancho, L.G., 2017. F_v/F_m acclimation to the Mediterranean summer drought in two sympatric *Lasallia* species from the Iberian mountains. The Lichenologist 49, 157-165.

Walters, P.A., Abbott, E.A., Isquith, A.J., 1973. Algicidal activity of a surface-bonded organosilicon quaternary ammonium chloride. Appl Environ Microbiol 25, 253-256.

Wessels, S., Ingmer, H., 2013. Modes of action of three disinfectant active substances: a review. Regul Toxicol Pharm 67, 456-467.

Young, M.E., Wakefield, R., Murquhart, D.C.M., Nicholson, K., Tonge, K. (1995). Assessment in a field setting of the efficacy of various biocides on sandstone. In: Methods of evaluating products for the conservation of porous building materials in monuments: preprints of the international colloquium (Rome, 19-21 June 1995). ICCROM, Rome, 93-99.

Figure captions

Fig. 1. Maximum quantum efficiency of Photosystem II photochemistry (F_V/F_M) in thalli of *Xanthoparmelia tinctina* measured one day (T1) after the application, with brush (left box-plots) and cellulose poultice (right box-plots), of water (white box-plots; negative control), BAC (light grey) and OIT-IPBC (dark grey), coupled or not with pre-hydration (non pre-hydrated, D; pre-hydrated, H) and/or washing (non washed, NW; washed, W) of thalli. Box-plots which do not share at least one letter are statistically different (ANOVA, Tukey's test, $p<0.05$). F_V/F_M values significantly lower than a viability threshold fixed at 0.15 (horizontal dotted line) are marked (*; ANOVA, *t*-test; $p<0.05$).

Fig. 2. Maximum quantum efficiency of Photosystem II photochemistry (F_V/F_M) in thalli of *Xanthoparmelia tinctina* measured one day before the application of BAC with cellulose poultice (T0), immediately after the poultice removal (T4h) and one day after (T1), coupled or not with pre-hydration and/or post-treatment washing of thalli (codes as in Fig. 1). At each time point, box-plots related to thalli pre-hydrated (H) or not pre-hydrated (D) before the biocide application which do not share at least one letter are statistically different (ANOVA, *t*-test, $p<0.05$). F_V/F_M values which are significantly lower than a viability threshold fixed at 0.15 (horizontal dotted line) are marked (*; ANOVA, *t*-test; $p<0.05$). Thalli on which the fluorimetric measurements were performed avoiding the usual moistening step are indicated (#).

Fig. 3. BAC in thalli of *Xanthoparmelia tinctina* after the application with brush and cellulose poultice in situ (four left columns) and with cellulose poultice in the laboratory (two right columns). Measures (mean ±SE) deal with non pre-hydrated thalli (D) and pre-hydrated thalli (H), which were washed (W) or not (NW) four hours after the biocide application. Separately considering *in situ* and laboratory assays, bars which do not share letters are significantly different (ANOVA, Tukey's test, $p<0.05$).

Fig. 4. BAC absorbed by absorbent paper -used to simulate a lichen thallus- after its desorption from the Macigno sandstone, used in the Amphitheatre of Luni, and from other sandstones for comparison (Pietra Serena, PS; sandstone of Cortemilia, CS; sandstone of the Verrucano Lombardo Formation, VL). Data are expressed as percentage of the amount of benzalkonium chloride (7.5 mg) initially applied on the examined parcels (mean±SE). Bars which do not share letters are significantly different (ANOVA, Tukey's test, $p<0.05$).

Fig. 5. Synoptic comparison of the influence of different application protocols on the efficacy of biocide treatments against the foliose lichen *Xanthoparmelia rinctina* (F_V/F_M at T1 was, ∨, or was not, ×, significantly lower than the vitality threshold set at 0.15).

669 **Tables**

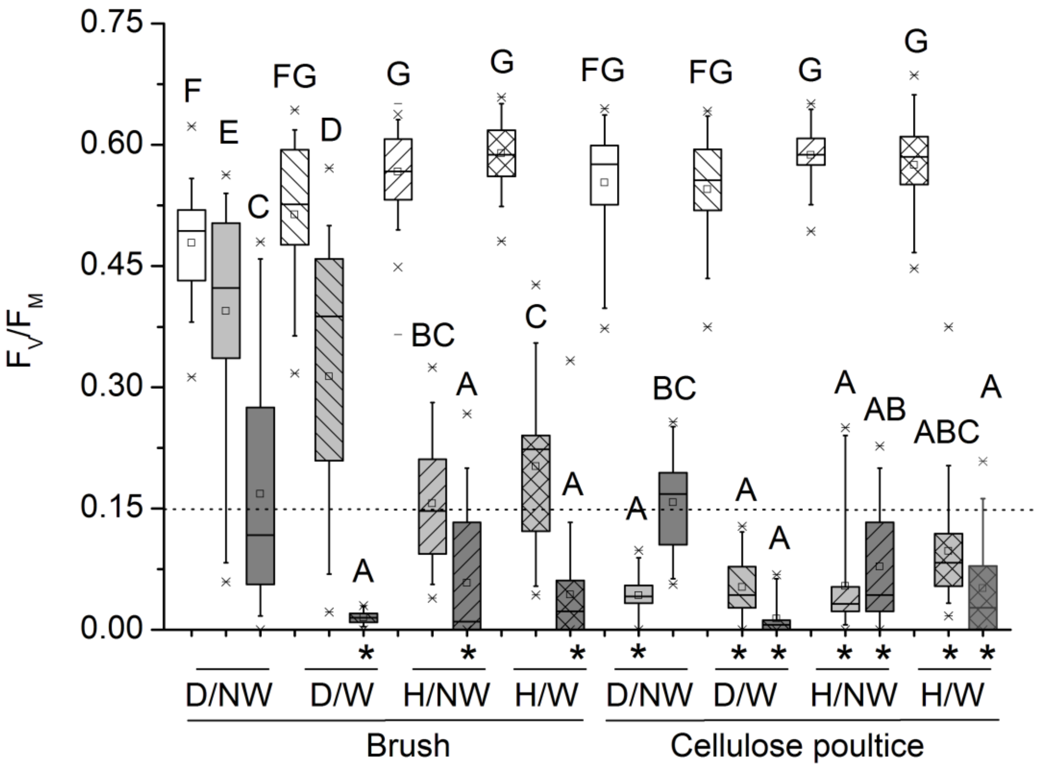
670 Table 1. Summary of the Generalized Linear Model

Parameter	Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
A) F_V/F_M	Product	32.029	2	16.015	196.125	**0.000**
	Appl. Tool	2.912	1	2.912	35.659	**0.000**
	Pre-Hydration	0.281	1	0.281	3.438	0.064
	Washing	1.659	1	1.659	20.317	**0.000**
	Error	55.852	684	0.082	-	-
B) F_0	Product	149018.955	2	74509.478	4.629	**0.010**
	Appl. Tool	248020.760	1	248020.760	15.410	**0.000**
	Pre-Hydration	99353.046	1	99353.046	6.173	**0.013**
	Washing	126628.182	1	126628.182	7.868	**0.005**
	Error	1.10090E+07	684	16095.073	-	-

671

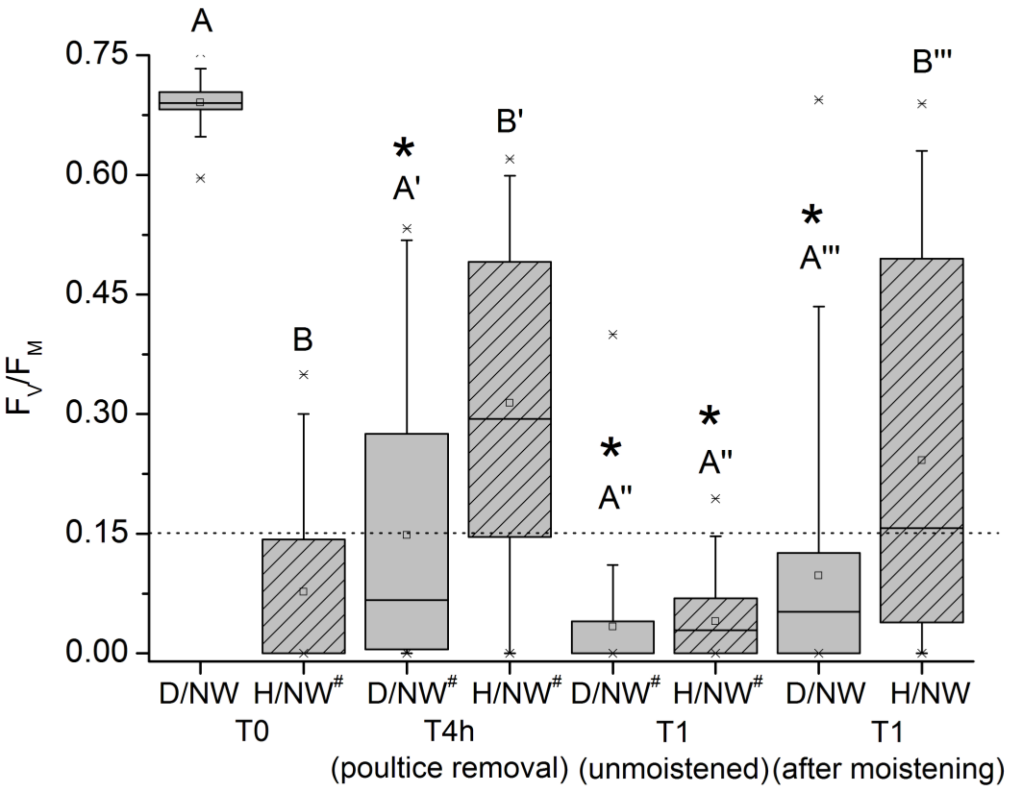
672

Fig. 1



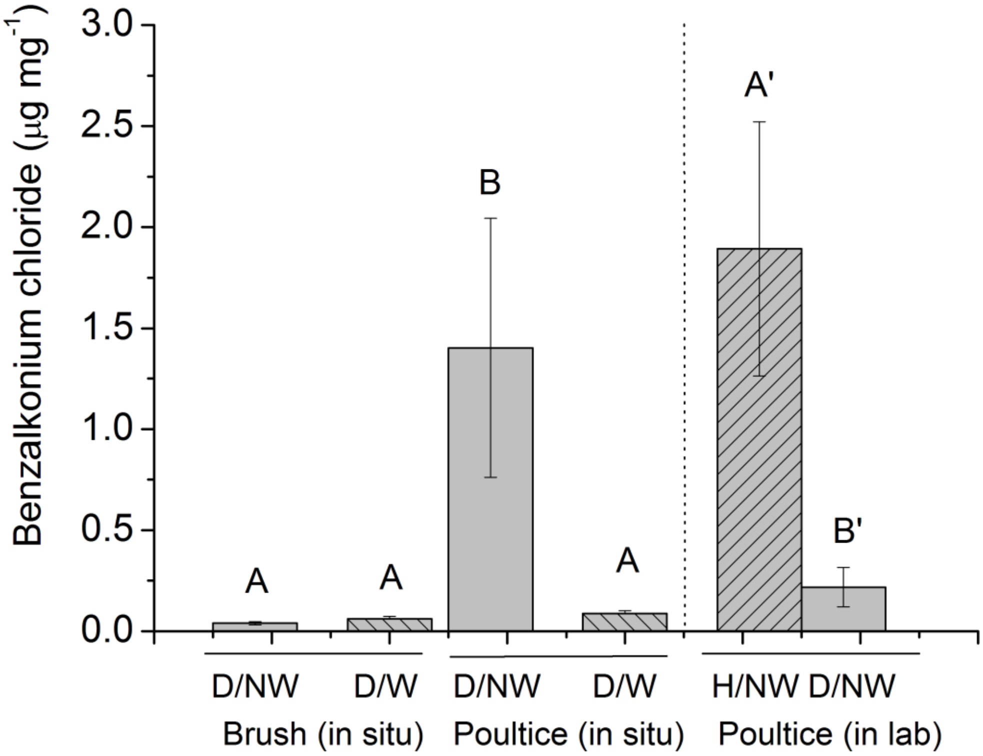
0.75
0.60
0.45
0.30
0.15
0.00
FV/FM
F
E
C
FG
D
A
G
BC
A
G
C
A
FG
A
BC
FG
A
A
G
A
AB
G
ABC
A
D/NW
D/W
H/NW
H/W
D/NW
D/W
H/NW
H/W
Brush
Cellulose poultice

Fig. 2



A
B
*
A'
B'
*
A''
*
A''
*
A'''
B'''
0.75
0.60
0.45
0.30
0.15
0.00
F_V/F_M
D/NW
H/NW#
D/NW#
H/NW#
D/NW#
H/NW#
D/NW
H/NW
T0
T4h
(poultice removal)
T1
(unmoistened)
T1
(after moistening)

Fig. 3



Benzalkonium chloride (µg mg-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
A
B
A
A'
B'
D/NW
D/W
D/NW
D/W
H/NW
D/NW
Brush (in situ)
Poultice (in situ)
Poultice (in lab)

Fig. 4

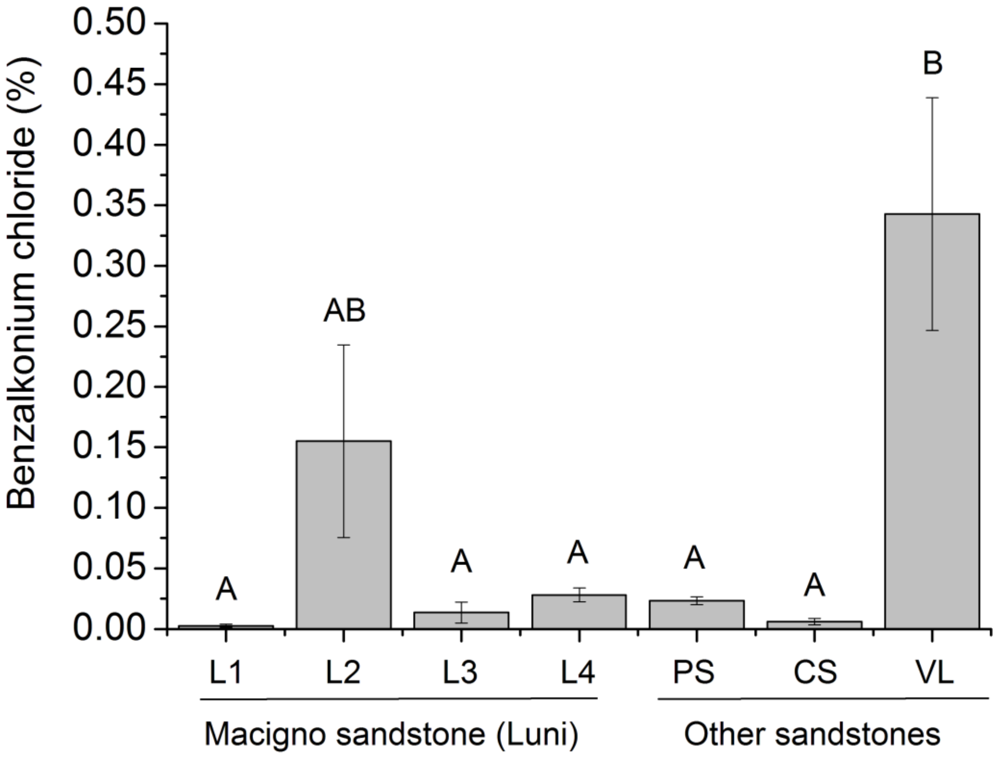
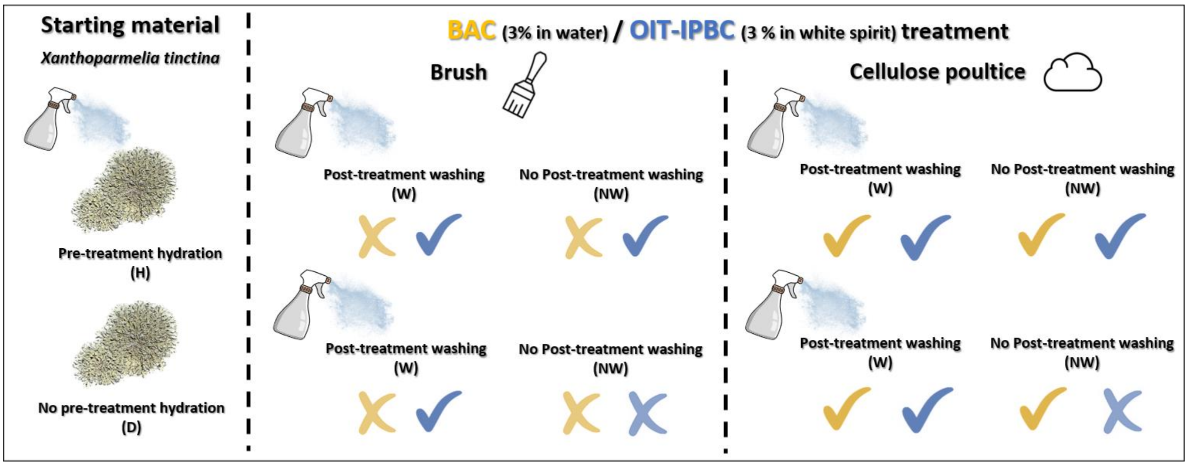

Benzalkonium chloride (%)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A
AB
A
A
A
A
B
L1
L2
L3
L4
PS
CS
VL
Macigno sandstone (Luni)
Other sandstones

Fig. 5


Starting material
Xanthoparmelia tinctina
Pre-treatment hydration
(H)
No pre-treatment hydration
(D)
BAC (3% in water) / OIT-IPBC (3 % in white spirit) treatment
Brush
Cellulose poultice
Post-treatment washing
(W)
No Post-treatment washing
(NW)