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This is the author's manuscript						
Original Citation:						
Availability:						
This version is available http://hdl.handle.net/2318/1758707 since 2020-10-19T12:39:52Z						
Published version:						
DOI:10.1016/j.ibiod.2020.105105						
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1 The application protocol impacts the effectiveness of biocides against lichens

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37 Abstract

This work analyzed the influence of different application protocols on the efficacy of two 38 biocides against the foliose lichen Xanthoparmelia tinctina on the sandstones of the Roman 39 40 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 41 devitalization success were verified by monitoring chlorophyll a fluorescence of thalli, both 42 43 in situ and in laboratory conditions. The hypothesis that (d) stone substrate may act as 44 reservoir for later biocide release under repeated cycles of wetting and drying was also 45 assayed. Analyses confirmed the importance of the application tool, with cellulose poultice being more effective than brush. Hydration influenced the biocide absorption by thalli. 46 Moreover it modulated the metabolic activity and susceptibility to the available toxic 47 48 compound, hindering lichens from entering a dormant state to tolerate stress. Depending on 49 the preparation solvent (water vs. white spirit), the biocide application benefited from pretreatment hydration and/or a post-treatment washing. Lastly, we showed that different 50 sandstones variously adsorb the biocides and potentially contribute as a reservoir for their 51 long-term release at low concentrations during successive hydration events. 52

53

54 Keywords

55 benzalkonium chloride, chlorophyll *a* fluorescence, lichen, thallus hydration, stone 56 conservation

57

58 Highlights

59 The protocol to apply biocides on lichens affects the devitalization effects

60 Cellulose poultice application of biocides is more effective than that by brush

61 Pre-hydration and/or post-washing of thalli regulate biocide effectiveness

62 The stone substrate acts as a reservoir for long-term release of biocide

63 Effective application protocols can limit useless chemical release to the environment

65 1. Introduction

The growth of lithobiontic (micro-)organisms widely affects the aesthetic and threatens the 66 durability of heritage surfaces (Caneva et al. 2008; Negi and Sarethy 2019). In particular, 67 lichens are primary agents of stone biodeterioration. Their metabolites induce mineral 68 leaching and biomineralization, and their hyphal penetration promotes disaggregation 69 processes (Adamo and Violante 2000; Favero-Longo et al. 2005; Seaward 2015). Despite 70 some bioprotective effects are recognized for certain species on certain lithologies (Salvadori 71 72 and Casanova-Municchia 2016), the removal of lichens is generally considered pivotal to 73 preserve heritage surfaces and is standard practice in conservation and restoration plans 74 (Pinna 2017).

75 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. 76 Tretiach et al. 2012; Mascalchi et al. 2015; Sanz et al. 2015; Rivas et al. 2018). Nevertheless, 77 78 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 79 including devitalization of thalli by biocide application, followed by their removal by 80 mechanical methods, are still commonly used by restorers (Kakakhel et al. 2019). Killing 81 lichens prior to their brushing or scraping from the stone surfaces is recognized as a crucial 82 need to prevent the persistence of viable thalline fragments within rock fissures and the 83 dispersal of propagules, which may promote rapid recolonization processes (Pinna 2017). 84 However, the effectiveness of biocidal treatments against lichens is not generalizable, and 85 86 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 87 has been demonstrated that the effectiveness of biocidal products is species- and site-specific 88 and it is strongly influenced by the application tools adopted (Favero-Longo et al. 2017). In 89 situ preliminary assays are thus necessary to evaluate the site- and species-specific 90 91 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. 92 2017; Pinna 2017). Certain practical steps of biocide application, which may affect their 93 effectiveness, are similarly worthy of investigation to validate protocols ensuring the 94 95 devitalization success.

96 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 97 strict relation to microenvironmental variation, the susceptibility of lichens to stress factors 98 depends on their hydration state. They are stress-tolerant when dry, while highly sensitive 99 when hydrated (even partially) and thus metabolically active (Tretiach et al. 2012). However, 100 the choice of applying biocides on previously hydrated or dry thalli is still a controversial 101 issue. Two contrasting hypotheses have been formulated, postulating that the pre-hydration of 102 lichen thalli may assist the biocide absorption or, oppositely, that it may favour a quicker 103 washing off and reduce absorption (Nugari and Salvadori 2003; Pinna 2017). Nevertheless, to 104 105 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. 106

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) 110 pre-treatment hydration step, and (c) post-treatment washing may, either singularly or in 111 combination, affect the effectiveness against lichens of biocides having different active 112 principles and dilution solvents. In particular, the effectiveness of different biocide treatments 113 against a foliose lichen, performed both in an archaeological site and in laboratory conditions, 114 was tested in terms of chlorophyll a fluorescence of the thalli with respect to a vitality 115 threshold ($F_V/F_M = 0.15$; Favero-Longo et al. 2017). We also verified the additional 116 hypothesis that (d) stone substrate may act as reservoir for later biocide release under repeated 117 118 cycles of wetting and drying.

119

120 2. Materials and methods

121 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).

134

135 2.2. *Biocide application* in situ

Benzalkonium chloride (BAC) as 3% water solution of Preventol RI80 (alkyl dimethyl benzyl 136 ammonium chloride, approx 80%, and isopropyl alcohol, 2%, in water; Lanxess, Köln, 137 Germany), and N-octyl-isothiazolinone and 3-iodo-2-propynyl-N-butylcarbamate (OIT-IPBC) 138 as 3% solution of BiotinR (OIT, 3-5%, and IPBC, 10-25%, in diethylene glycol butyl ether; 139 CTS, Altavilla Vicentina, Italy) in white spirit (Kelix, Thormax Italia, Roma) were selected as 140 biocides. They were applied either (i) using a paint-brush or (ii) with a cellulose poultice 141 (Arbocel BC 1000, JR Pharma, Rosenberg, Germany), (i') after having moistened the thalli 142 with sprayed water or (ii') avoiding this pre-hydration step. Per each surface unit of thallus, 143 brush applications required approx. 0.3 mL cm⁻² of diluted biocides; the applied poultice 144

layer, approx. 1 cm thick, contained approx. 12 mL cm⁻³. The cellulose poultice was covered 145 with a cotton fabric for 4 h and later gently removed with a small spatula, thereafter (i'') 146 washing the thalli or (ii'') avoiding this washing step. Thalli treated with water only in place 147 of biocides were assayed as negative controls. Three thallus replicates per biocide per 148 application method were examined [i.e. 3 replicates \times (2 biocides + 1 control) \times 2 application 149 tools \times 2 pre-treatment approaches \times 2 post treatment approaches]. Treatments including the 150 151 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 152 biocide dilution and the pre-hydration and washing steps. 153

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).

157

158 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.

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166 *2.4. Lichen vitality measurements*

Chlorophyll a fluorescence measurements (Chl_aF) - recognized as a tool for checking the 167 vitality of photosynthetic organisms (Tretiach et al. 2008) - were carried out on X. tinctina in 168 situ one day before (T0) and one day after (T1) biocide treatments, using a Handy-PEA 169 fluorimeter (Plant Efficiency Analyser, Hansatech instruments Ltd., Norfolk, England), . 170 Analyses were performed on dark-adapted thalli, covered overnight with a black cotton fabric, 171 which were moistened by sprayed water just before the measurements, to avoid that the 172 additional hydration may further affect the biocide action. Measurements on the thalli treated 173 in the laboratory were carried out one day before the biocide application (T0), immediately 174 after the removal of cellulose poultice (T4h) and one day after (T1). Analyses were performed 175 following the protocol adopted in situ, with the exception that the moistening at T0 was 176 avoided for thalli foreseen without the pre-hydration step, and that measurements at T1 were 177 178 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 temperatureindependent 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).

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189 2.5. Biocide absorption by lichen thalli

190 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 191 laboratory, were gently detached from the rock substrate with a scalpel and processed to 192 analyse the absorbed BAC. In particular, they were carefully cleaned under a 193 stereomicroscope and then left overnight in a climatic chamber at 16°C and 55% of relative 194 humidity (residual water content <10%). Samples of 50 mg were homogenized with 1 mL of 195 deionized water and centrifuged at 20,000 rfc for 10 min. The supernatant was filtered at 0.45 196 um using a syringe filter and 30 µL of the solution were directly analyzed by HPLC (Water 197 198 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 199 phase with flow rate 1 mL/min (Rojsitthisak et al. 2005). Runs were monitored at 210nm. 200 Quantification was performed with a calibration curve $(5 - 50 \mu g/mL)$ of BAC from Sigma-201 Aldrich (\geq 95.0%). The limit of quantification of the analysis was 0.04 µg mg⁻¹. 202

203

204 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 205 on the rock surface and a subsequent washing with deionized water was assessed in the 206 207 laboratory. In particular, four sandstone blocks of the Macigno Formation, similar to those 208 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 209 saw (section thickness >5 cm). In the central parts of the cut surfaces, parcels (2×3 cm) were 210 211 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 212 let to dry overnight at room temperature. Thereafter, 250 µL of deionized water were applied 213 on each parcel and (after 30 seconds) a double layer of absorbent paper (9 mg cm^{-2}) was 214 applied -to simulate the potential absorption of a lichen thallus- and let dry on the rock 215 surface. The absorbent paper was then suspended in 2 mL of deionized water to extract BAC, 216 which was quantified as described above. Blocks of other sandstone lithologies employed in 217 the Italian stone cultural heritage were also cross-sectioned and similarly processed for 218 219 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 220 Verrucano Lombardo Formation, well known for rock-art in the Valle Camonica (Brack et al. 221 2008). At least three parcels per treatment (BAC, water) were considered per each block. 222 Moreover, the same process was repeated on glass slides, as negative control. 223

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).

230

231 *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 prehydration 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 239 240 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 241 means of ANOVA with post-hoc Tukey's and t-test, respectively, using SYSTAT 10.2 242 (P<0.05 as significant). For each study case, significant differences of F_0 in the thalli treated 243 with biocides with respect to the control ones were assessed at T1. Significant differences in 244 the absorption of BAC by lichen thalli, and in the BAC desorption patterns by different 245 sandstone lithologies, were also examined by means of ANOVA with Tukey's post-hoc test. 246

247

248 **3. Results**

249 *3.1. Efficacy of devitalization treatments* in situ

GLM analyses (Table 1) showed that all the considered factors (product, application tool, prehydration 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.
- F₀ values (Figs. S2-S3) strongly decreased with respect to controls only for OIT-IPBC application on pre-hydrated thalli (mean \pm SE: -62 \pm 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%).

265

266 *3.2. Efficacy of devitalization treatments in the laboratory*

267 In the laboratory, the application of BAC with cellulose poultice was effective against thalli 268 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 269 270 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 271 cellulose poultice (T4h), without any additional moistening, F_V/F_M of thalli treated in the wet 272 state was significantly lower than the vitality threshold and with respect to thalli treated in the 273 dry state. At T1, all thalli were dehydrated and F_V/F_M was significantly below 0.15, but after 274 their moistening, those which had received the poultice application in the dry state recovered 275 276 F_V/F_M values significantly higher than the threshold.

277

278 *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 μ g mg⁻¹) with respect to those washed after the poultice removal and those treated with brush (0.1 μ g 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 μ g 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 μ g mg⁻¹).

292 *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 ofmagnitude higher, above 30%.

On the basis of SEM-BSE observations (Fig. S4), the Verrucano Lombardo showed an 300 301 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 302 which it was mobilized during the subsequent re-wetting. Oppositely, in the case of the other 303 sandstones, the applied biocide clearly entered the rock volume. In the case of the Macigno 304 305 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 306 a fitted fabric due to pressure dissolution, with juxtaposed grains and absence of cement or 307 matrix, while the Cortemilia sandstone showed traces of carbonate cement and a clay fraction. 308 A fine-grained sericitic matrix possibly characterized the block of Verrucano Lombardo. 309

310

311 **4. Discussion**

Our findings support the hypothesis that the protocol adopted to apply biocides significantly 312 affects the devitalization of lichen thalli (Fig. 5). Besides confirming the importance of the 313 application tool, with cellulose poultice being more effective than brush (Favero-Longo et al. 314 2017; Matteucci et al. 2019), this experimental work clarified the remarkable influence of the 315 state of hydration of lichen thalli on their susceptibility to biocides. Hydration modulates the 316 biocide absorption by thalli. Moreover, it controls their maintaining an active metabolism or 317 318 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 319 biocides, we highlighted the biocide-specific advantage of pre-treatment hydration and/or 320 post-treatment washing of thalli to improve the application protocol effectiveness. In 321 particular, the poultice application was necessary to make effective against X. tinctina the 322 assayed water-solution of benzalkonium chloride (BAC), independently of the pre- or post-323 treatment hydration of thalli. Differently, the washing of thalli after the biocide application 324 was necessary to make effective the assayed organic-solvent solution of N-octyl-325 326 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 327 biodeteriogens will be routinely available, the conventional use of traditional biocides by 328 restorers cannot overlook this necessity of adopting effective application protocols and hence 329 limit the useless release of biocides in the environment. In addition, this work showed that the 330 331 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 332 successive hydration events. 333

334 *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 non-

ezymatic mechanisms to protect the integrity of cellular components and limit pro-oxidative 338 processes (Kranner et al. 2008), an effective machinery to maintain proteostasis (Armaleo et 339 al. 2019) and the interplay of the whole lichen microbiota (Cernava et al. 2019). A notable 340 example is the tolerance to high temperatures, which for dry thalli ranges from 70°C to more 341 than 100°C depending on species (Lange 1953), while it is generally lower than 45-50°C 342 343 when thalli are forcedly maintained in the hydrated state (McFerlane and Kershaw 1978; 344 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 345 which the symbionts are metabolically active and sensitive to their toxic effects (Vannini et 346 al. 2020). 347

A similar pattern is here confirmed for the foliose lichen X. tinctina treated with the water 348 soluble BAC and OIT-IPCB prepared in white spirit. The quaternary ammonium salt BAC 349 perturbs the phospholipid bilayer of the biological membranes, causing their damage and the 350 cell lysis (Wessels and Ingmer 2013). OIT oxidazes thiol-containing cytoplasmic and 351 membrane-bound compounds, yielding metabolic inhibition (Denyer and Stewart 1998), and 352 IPBC disrupts the formation of fungal cell walls by interfering with synthesis of 353 phospholipids and fatty acids (Biehl 2019). Despite their different active principles, target 354 molecules and solubility, thallus hydration influences the effectiveness of both products. 355

The poultice application of BAC in situ, which always decreased F_V/F_M below the viability 356 threshold set at 0.15, carried the water-dissolved biocide as well as contributed to maintain 357 wet the pre-hydrated thalli and to hydrate the thalli in the dry state (Favero-Longo et al. 358 359 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 360 (T4h), indicating its metabolic activation by water rather than its devitalization by the biocide. 361 The dry state at the time of the application also implied in the laboratory a significantly lower 362 363 BAC absorption with respect to that detected in the pre-hydrated thalli. Such findings agree 364 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 365 reject the hypothesis that pre-hydration may reduce the absorption of biocides (Tretiach et al. 366 2007). 367

368 In situ, BAC absorption in non pre-hydrated thalli was instead more similar to that of the prehydrated thalli in the laboratory. With this regard, it is worth noting that F_V/F_M of lichen 369 thalli, and thus their metabolic activity, is also highly related to weather conditions during the 370 two days prior to the measurements (Vivas et al. 2017). Differing from the thalli kept in the 371 laboratory in the dry state, those in the study site were regularly exposed to high humidity 372 levels during the night (RH above 80-90% in the days before the treatment; Fig. S1). 373 Although they were dried at the time of biocide application (around noon), they likely had a 374 higher attitude, in terms of physiological state, to recover their metabolic activity by 375 effectively absorbing the water solution of BAC, even without the pre-treatment hydration 376 377 step.

The application of BAC by brush contributed a lower quantity of biocide and did not maintain 378 the hydration of the thalli, justifying a lower absorption and the poor devitalization 379 effectiveness. The fact that post-treatment washing did not lead to a further lowering of F_V/F_M 380 suggests that the effect was likely more limited by the biocide quantity than by a scarce 381 metabolic activation. This also agrees with the fact that the absorbed BAC did not decrease 382 with the post-washing step, suggesting that the available biocide had been effectively 383 384 absorbed and retained by the cell structures. In this sense, neither brush nor poultice application determined at T1 a remarkable decrease of F₀, detectable upon the loss of 385 chlorophyll following membrane integrity impairment (Vannini et al. 2018), suggesting that 386 BAC-driven cell lysis had still not deeply proceeded and that some absorbed BAC could not 387 388 be washed away.

In the case of OIT-IPBC, no difference was detected in the effectiveness of the assayed 389 application tools, indicating that the lower quantity of active principles carried by the brush 390 was sufficient to kill the lichens. However, for this biocide prepared in white spirit, the 391 wetting of thalli by the pre-treatment hydration and/or the post-treatment washing was a 392 necessary requirement to make the treatment effective. Accordingly, the removal of crustose 393 and foliose lichens following the application of BiotinR with a protocol which does not 394 mention hydration steps determined the persistance of thallus remains with some (few) viable 395 photobiont cells (de los Ríos et al. 2012). As hypothesized for other biocides, but not 396 experimentally verified (Tretiach et al. 2007), the post-treatment washing of thalli treated 397 398 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 399 method to favour its susceptibility and face its defence strategies. This agrees with the report 400 that IPBC is highly soluble in organic solvents and poorly soluble in water (156 mg L^{-1} at 401 20°C; Juergensen et al. 2000), but its efficacy depends on the water dissolved fraction and its 402 general wide use is related to strategies to allow its dissolution, including the predissolution in 403 organic solvents (Steinberg 2002). However, the highest decrease of F_0 (>60%) was observed 404 in thalli pre-hydrated, either washed or unwashed, suggesting that they mostly faced a strong 405 damage of cell structures and the damage and loss of chlorophyll. In thalli treated when dry, 406 instead, F₀ showed a relative increase, which may reflect the initial presence of some free 407 chlorophyll due to membrane damage (Strasser 1997), or a resistance attempt towards a 408 treatment with incomplete killing efficacy (Favero-Longo et al. 2017). Further investigations 409 will be necessary to clarify such response patterns of thalli as well as to unveil if and how 410 411 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 412 already revealed different sensitivity to different biocidal products (de los Ríos et al. 2012) 413 and play a crucial role in biodeterioration processes (Speranza et al. 2012). 414

415

416 *4.2. Does substrate porosity influence the biocide efficacy?*

The different effectiveness of biocides against the growth of algae inoculated on sandstonelithologies 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 419 minerals or be slowly desorbed and become available to (micro-)organism absorption under 420 repeated cycles of wetting and drying (Cameron et al. 1997). For some biocides, the 421 adsorption to clay minerals may determine their inactivation, but quaternary ammonium salts 422 should maintain their biocidal activity when bound (Walters et al. 1973; Cameron et al. 1997). 423 424 These processes, however, have been infrequently investigated (Koestler and Salvadori 1996) 425 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 426 carefully considered with respect to the widespread application of biocides as a preventive 427 tool to protect heritage surfaces from recolonization process, which is to maintain rock 428 cleaning after the removal of lichens and biofilms (Pinna 2017). 429

430 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 431 biocide application and consequent rock adsorption, depends on physical and mineralogical 432 properties of the different sandstone lithologies. In particular, the higher the rock porosity and 433 the presence of clay minerals, the lower the biocide desorption at new rain events or watering. 434 In the case of lithologies with very low porosity, such as Verrucano Lombardo, the biocide 435 visibly crystallized at the rock surface and could likely be washed off by flowing water rather 436 than persist as long-term protection (Cameron et al. 1997). Even within the same lithology, 437 clay contents can vary from a block to another, as in the case of the Macigno sandstone in 438 439 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 2-440 3 orders of magnitude lower than that provided during the application, which turned effective 441 442 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 443 abundantly bound within the rock porosity (Cameron et al. 1997), although the bio-activity 444 should be demonstrated for each considered quaternary ammonium compound. On the other 445 hand, such application strategy produces a reservoir for their gradual release at low and likely 446 ineffective concentrations. With this regard, the phenomenon may be likely related to the 447 reported cases of surface eutrophication following the application of quaternary ammonium 448 salts, their degradation and consequent nitrogen supply, favouring recolonization processes by 449 nitrophilous, fast growing species (Scheerer et al. 2009). Moreover, the release of low and 450 ineffective concentrations of BAC can promote bacterial adaptation and antibiotic resistance 451 452 (Kampf 2018; Kim et al. 2018; Poursat et al. 2019).

453

454 **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).

459 Acknowledgements

- 460 This work is part of the project "Licheni e Beni Culturali in Pietra Adotta un Monumento",
- 461 carried out by the Working Group for Biology of the Italian Lichen Society and financially
- 462 supported by Istituto Superiore per la Conservazione ed il Restauro, Roma. The authors are
- grateful to Marcella Mancusi (Polo Museale della Liguria) and the staff of the Archaeological
- site of Luni for assistance during field activities, to Leonardo Borgioli (CTS, Altavilla
- 465 Vicentina) for providing BiotinR, and to Eraldo Bocca and Cinzia Morachioli (Lievito Madre
- 466 A.P.S.) for their kind hospitality in Castelnuovo Magra during the Working Group workshops
- 467 in April 2018 and May 2019.
- 468

469 **References**

- Adamo, P., Violante, P., 2000. Weathering of rocks and neogenesis of minerals associated
 with lichen activity. Appl Clay Sci 16, 229-256.
- 472 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.
- 481 Baruffo, L., Tretiach, M., 2007. Seasonal variations of F_0 , F_M , and F_V/F_M in an epiphytic 482 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,
- 490 Monte Adamello. Carta Geologica d'Italia alla scala 1: 50.000. ISPRA, Roma.
- 491 Cameron, S., Urquehart, D., Wakefield, R., Young, M., 1997. Biological growths on
- 492 sandstone buildings. Control and treatment. Technical advice note, 10. Historic Scotland
- 493 (Technical Conservation, Research Education Division), Edimburgh.
- 494 Caneva, G., Nugari, M.P., Salvadori, O., (Eds.), 2008. Plant biology for cultural heritage:
 495 biodeterioration and conservation. Getty Publications, Los Angeles.

- 496 Cernava, T., Aschenbrenner, I. A., Soh, J., Sensen, C. W., Grube, M., Berg, G., 2019.
- 497 Plasticity of a holobiont: desiccation induces fasting-like metabolism within the lichen498 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.
- 510 Favero-Longo, S.E., Benesperi, R., Bertuzzi, S., Bianchi, E., Buffa, G., Giordani, P., Loppi,
- 511 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.
- 514 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,
- 516 B.E. (Eds.), Global heritage stone: Towards international recognition of Building and
- ornamental stone. Geological Society, London, Special Publications, 407: 173-186.
- 518 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.
- 521 Gelati, R., Gnaccolini, M., Polino, R., Mosca, P., Piana, F., Fioraso, G., 2010. Note
- 522 illustrative della Carta Geologica d'Italia alla scala 1:50000, Foglio 211 Dego. ISPRA-
- 523 Istituto Superiore per la Protezione e la Ricerca Ambientale, pp. 117.
- 524 Giordani, P., Nicora, P., Rellini, I., Brunialti, G., Elix, J. A. (2002). The lichen genus
- 525 *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.
- 528 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 BiodeteriorBiodegrad 143, 104721.
- 531 Kampf, G., 2018. Adaptive microbial response to low-level benzalkonium chloride exposure.
- 532 J of Hosp Infect 100, e1-e22.

- 533 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.
- 540 Kylin, H., Bouwman, H., 2012. Hydration state of the moss *Hylocomium* splendens and the
- 541 lichen *Cladina stellaris* governs uptake and revolatilization of airborne α -and γ -542 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.
- 545 Malaspina, P., Giordani, P., Faimali, M., Garaventa, F., Modenesi, P., 2014. Assessing
- 546 photosynthetic biomarkers in lichen transplants exposed under different light regimes.
- 547 Ecological Indicators 43, 126-131.
- 548 Mascalchi, M., Osticioli, I., Riminesi, C., Cuzman, O.A., Salvadori, B., Siano, S., 2015.
- 549 Preliminary investigation of combined laser and microwave treatment for stone
 550 biodeterioration. Stud Conserv 60(Supplement 1), 19–26.
- 551 Matteucci, E., Scarcella, A.V., Croveri, P., Marengo, A., Borghi, A., Benelli, C., Hamdan, O.,
- 552 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
- 555 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.
- 560 Nugari, M.P., Salvadori, O., 2003. Biocides and treatment of stone: limitations and future
- 561 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
- 566 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
- 568 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,
- 572 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.
- 575 Rojsitthisak, P., Wichitnithad, W., Pipitharome, O., Sanphanya, K., Thanawattanawanich, P.,
- 576 2005. Simple HPLC determination of benzalkonium chloride in ophthalmic formulations
 577 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.
- 581 Salvadori, O., Charola, A.E., 2011. Methods to prevent biocolonization and recolonization: an
- 582 overview of current research for architectural and archaeological heritage. In: Charola A.E.,
- 583 McNamara, C., Koestler, R.J. (Eds), Biocolonization of stone: Control and preventive
- 584 methods. Proceedings from the MCI Workshop Series. Smithsonian Contributions to
- 585 Museum Conservation (Vol 2). Smithsonian Inst. Press, Washington, 37-50.
- 586 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
 Diadatarian Diadataria 142, 151, 150
- 589 Biodeterior Biodegrad 142, 151-159.
- 590 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.
- 596 Seaward, M.R.D., 2015. Lichens as agents of Biodeterioration. In Recent Advances in
- 597 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.
- 600 Speranza, M., Wierzchos, J., De Los Rios, A., Perez-Ortega, S., Souza-Egipsy, V., Ascaso,
- 601 C., 2012. Towards a more realistic picture of in situ biocide actions: Combining
- 602 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.
- 613 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.
- 616 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 overtime? 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.
- 629 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.

634 Figure captions

- Fig. 1. Maximum quantum efficiency of Photosystem II photochemistry (F_V/F_M) in thalli of
- 636 *Xanthoparmelia tinctina* measured one day (T1) after the application, with brush (left box-
- 637 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-
- 639 hydrated, D; pre-hydrated, H) and/or washing (non washed, NW; washed, W) of thalli. Box-
- 640 plots which do not share at least one letter are statistically different (ANOVA, Tukey's test,
- 641 p<0.05). F_V/F_M values significantly lower than a viability threshold fixed at 0.15 (horizontal
- 642 dotted line) are marked (*; ANOVA, *t*-test; p<0.05).
- 643 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
- 647 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,
- 649 *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
- 652 (#).

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 \pm 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

- 658 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 \pm SE). Bars which do not share letters are significantly different (ANOVA, Tukey's test, p<0.05).
- which do not share letters are significantly different (AIVOVA, 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, \lor , or was not, \times , significantly lower than the vitality threshold set at 0.15).

Tables

Parameter	Source	Sum-of- Squares	df	Mean- Square	F-ratio	Р
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	-	-

Table 1. Summary of the Generalized Linear Model















685 Fig. 5

