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Title	Medium-term field evaluation of several widely used cleaning-restoration techniques applied to algal biofilm formed on a granite-built historical monument
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Abstract

This study evaluated the medium-term performance of some of the most widely used methods of cleaning stone: dry brushing or washing with water or with an ethanol-water (1:1) mixture, benzalkonium chloride or commercial products based on quaternary ammonium salts with biocidal properties (Biotin R®, Biotin T®, Preventol RI80®, and New Des 50®). The ability of the different methods to remove an algal biofilm formed on a granite-built historical monument was compared. The biofilm has been shown to affect the appearance, but not the integrity, of the building material. In the cleaning trials, the presence and vitality of the organisms colonizing the substrate were determined, immediately after treatment and after one, three, six and twelve months, by colour spectrophotometry and chlorophyll-a fluorescence (ChlaF) analysis. The quantity and physiological state of the biofilm-forming phototrophs in the test areas were also determined one year after the treatments. Although recolonization of the test areas was barely noticeable after all of the treatments, Preventol RI80® proved to be the most effective, followed by benzalkonium chloride and the ethanol-water (1:1) mixture, which yielded similar results. New Des 50® caused clearly visible colour changes on the granite wall, and the biocidal efficacy was poor. Biotin R® performed better than Biotin T®, which yielded similar results to New Des 50® regarding recolonization. Considering the overall results and the potential toxicity of the products tested, cleaning with water is recommended if and when required.

Keywords	cleaning efficacy; commercial biocides; monitoring techniques; phototrophic colonization; recolonization; tertiary bioreceptivity.
Taxonomy	Stone, Biodeterioration, Damage to Buildings, Colonization
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Highlights

- Field application of common cleaning methods on granite-built heritage was evaluated
- Short and medium-term treatment efficacy was evaluated by *ChlaF* and colour analysis
- *Preventol RI80* yielded the best results, and *New Des 50* had a deleterious impact
- Biotin R® performed better than Biotin T®
- Ethanol (50%) and benzalkonium chloride were effective, especially in the short term

Medium-term field evaluation of several widely used cleaning-restoration techniques applied to algal biofilm formed on a granite-built historical monument

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1 **Abstract**

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3 methods of cleaning stone: dry brushing or washing with water or with an ethanol-water
4 (1:1) mixture, benzalkonium chloride or commercial products based on quaternary
5 ammonium salts with biocidal properties (Biotin R®, Biotin T®, Preventol RI80®, and
6 New Des 50®). The ability of the different methods to remove an algal biofilm formed
7 on a granite-built historical monument was compared. The biofilm has been shown to
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10 determined, immediately after treatment and after one, three, six and twelve months, by
11 colour spectrophotometry and chlorophyll-a fluorescence (ChlaF) analysis. The quantity
12 and physiological state of the biofilm-forming phototrophs in the test areas were also
13 determined one year after the treatments. Although recolonization of the test areas was
14 barely noticeable after all of the treatments, Preventol RI80® proved to be the most
15 effective, followed by benzalkonium chloride and the ethanol-water (1:1) mixture,
16 which yielded similar results. New Des 50® caused clearly visible colour changes on
17 the granite wall, and the biocidal efficacy was poor. Biotin R® performed better than
18 Biotin T®, which yielded similar results to New Des 50® regarding recolonization.
19 Considering the overall results and the potential toxicity of the products tested, cleaning
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22 techniques; phototrophic colonization; recolonization; tertiary bioreceptivity.

23

24 1. Introduction

25 Removal of colonizing organisms from the surface of historical monuments and
26 structures is controversial, as the effects of the colonizing communities (which may be
27 deteriorative, negligible or even protective) must be carefully considered and weighed
28 against aesthetic and site management factors (Pinna, 2014; Bartoli et al., 2014;
29 Salvadori and Casanova-Municchia, 2016; Caneva et al., 2018). The community
30 biodiversity and related factors must also be taken into account (Cicinelli et al., 2018,
31 2019), and the reality is that cleaning and conserving historical stone buildings is
32 generally a very complicated procedure. Indeed, the cleaning of heritage sites involves a
33 huge financial outlay (see e.g. Europanostr.org). According to the Santiago de
34 Compostela (Spain) city council, the cost of removing subaerial biofilms (SABs) from
35 the main facade (Obradoiro) of the Pazo de Raxoi (city council buildings) in 2016
36 amounted to €318,000. As this is a routine task carried out by restorers, conservators
37 and architects, detailed study of the effectiveness and impact of cleaning treatments is
38 very important in order to establish strategies of controlling or preventing biological
39 colonization on buildings.

40
41 The efficacy of cleaning treatments depends on a number of factors, such as the target
42 species, environmental conditions, mechanical removal strength and product application
43 (see e.g. Favero-Longo et al., 2017; Kakakhel and Wu et al. 2019). Product selection is
44 usually based on the experience of the restorers and the availability of commercial
45 formulations, supported by empirical evaluations that in some cases report negative side
46 effects, such as in the famous Lascaux Cave (Kakakhel and Wu et al. 2019).

47
48 On-site monitoring of treatment efficacy is an essential part of the intervention. Non-
49 destructive analysis to determine the biomass and physiological state of phototrophs is
50 ideal for monitoring purposes and is preferable to determining the phototrophic
51 components of subaerial biofilms (SABs) by destructive analysis. In this regard, colour
52 measurement expressed in the CIELAB space (CIE Publication 15-2, 1986) has been
53 firmly established as a proxy indicator for early quantification of phototrophic subaerial
54 biofilm formation on granite stone (e.g. Sanmartín 2012 and references therein;
55 Sanmartín et al., 2012; Pozo-Antonio et al., 2017). The technique is also suitable for
56 determining the response of phototrophic microorganisms to biocidal strategies, as L*
57 and a* CIELAB parameters are closely related to physiological parameters such as
58 photosynthetic pigments (chlorophyll-a, phaeopigments) and ATP content (Sanmartín et
59 al., 2011, 2015). Likewise, the chlorophyll-a fluorescence (Chl_aF) parameter F_v/F_m
60 (maximum quantum efficiency of PSII) has been reported to be suitable for ascertaining
61 the vitality of algal remains of subaerial biofilms on granite (Pozo-Antonio and
62 Sanmartín, 2018).

63
64 In this research, we evaluated the medium-term (one year) efficacy of some of the stone
65 cleaning methods currently most widely used by conservators and restorers, particularly
66 for cleaning granite-built heritage. The treatments, which include dry brushing, washing
67 with water, ethanol-water (1:1), benzalkonium chloride or with commercial products

68 based on quaternary ammonium salts with biocidal properties (Biotin R®, Biotin T®,
69 Preventol® RI80, and New Des® 50) were tested on a highly hydrophobic subaerial
70 biofilm, formed by *Apatococcus lobatus* (Chodat) J.B.Petersen (Chlorophyta), on the
71 processional cloister of the Monastery of San Martiño Pinario (Sanmartín et al., this
72 issue). In the cleaning trials, the presence and vitality of the organisms colonizing the
73 substrate were determined, immediately after cleaning and after one, three, six and
74 twelve months, by colour spectrophotometry and modulated chlorophyll-a fluorescence
75 analysis. The quantity and physiological state of biofilm-forming phototrophs on the
76 test areas were also analysed one year after the treatments.

77 **2. Materials and methods**

78 **2.1. Study area**

79
80 The target building under consideration is adjacent to the cathedral in the historical
81 centre of Santiago de Compostela (UNESCO World Heritage City since 1985, capital of
82 Galicia, NW Spain). The Monastery of San Martiño Pinario (UTM 29T X 537220, Y
83 4747793; Datum ETRS89, Figure 1), which was built between the 9th and 18th
84 centuries, has a distinguished architecture ranging from baroque to neoclassic, and is the
85 most important constructive complex in the city after the cathedral. It is also considered
86 the most valuable baroque building in Galicia. The procedure for recognizing the
87 Monastery as an Asset of Cultural Interest for the year 2021 (*Xacobeo*, Holy Year) was
88 initiated at the end of 2019.

89
90 The monastery's processional cloister of granite stonework, built between 1633 and
91 1747, has a traditional structure of sober classic lines, including four bays surrounding a
92 courtyard with a fountain in the centre (Figure 1). It has two floors - a high cloister and
93 a lower cloister - joined by paired columns of monumental Tuscan order, each set on a
94 high plinth. The first floor, or lower cloister, is articulated by means of half-point
95 arches, while the upper cloister has vaulted flat balconies, with pairs of large Doric
96 columns in the intermediate spaces. The whole building is enclosed by a bell tower.
97 Although the project is attributed to Bartolomé Fernández Lechuga, Peña del Toro and
98 Miguel de Romay were also involved, the latter as designer of the bell tower.

99
100 Further information about the granite masonry of the background walls of the cloister,
101 as well as the joint mortar, is reported in Sanmartín et al. (this issue) and references
102 therein. A comprehensive explanation regarding the colonization pattern outlined by the
103 subaerial biofilm growth through the background walls is also given in the
104 aforementioned paper.

105 106 **2.2. Cleaning treatments**

107
108 Eight adjacent areas on the west-facing background wall of the processional cloister of
109 the Monastery of San Martiño Pinario (biofilm cover) (Figure 2), all with the same level
110 (or degree of presence) of biological colonization, were selected according to previous

111 studies by Pozo-Antonio et al. (2017) and Pinna et al. (2018). In each trial area, one of
112 the 8 treatments was applied with a brush to the subaerial biofilm formed by
113 *Apatococcus lobatus* (Chodat) J.B.Petersen (Chlorophyta) (Sanmartín et al., this issue).
114 The biofilm was removed following the method described by Pozo-Antonio and
115 Sanmartín (2018). One untreated (colonized) area and one uncolonized area adjacent to
116 the treated areas were also analysed for reference purposes (Figure 2).

117

118 The 8 treatments were as follows: dry brushing; washing with water; washing with an
119 ethanol-water mixture (1:1), i.e. ethanol 50% (v/v in distilled water), as in previous
120 studies on granitic rocks (Pozo et al., 2013; Pozo-Antonio et al., 2017); washing with
121 benzalkonium chloride; and washing with Biotin R®, Biotin T®, Preventol RI80® or
122 New Des 50® (commercial products based on quaternary ammonium salts with biocidal
123 properties). Detailed descriptions of the six chemical products are provided in Table 1.

124

125 Each area to which each treatment was applied (single application) measured about
126 20×20 cm. At the end of the experiment, colour measurements were made (section 2.3)
127 and chlorophyll pigments were extracted (section 2.5) in the whole area, which was
128 divided into 3 replicate areas, each of ca.120 cm². Due to the small size (4 mm in
129 diameter) of the measuring head of the Pulse-Amplitude-Modulation (PAM)
130 chlorophyll fluorometer, a smaller, central area (of about 10×10 cm) in each test area
131 was also divided into 3 replicate areas (each of ca. 30 cm²) for chlorophyll fluorescence
132 measurement (section 2.4).

133

134 Recolonization was monitored in each trial area throughout a period of 12 months
135 (May/2018–May/2019), i.e. immediately after treatments (May 2018) and after one
136 month (June 2018), three months (August 2018), six months (November 2018) and
137 twelve months (May 2019) by colour spectrophotometry and modulated chlorophyll-a
138 fluorescence analysis. The chlorophyll-a content and phaeophytization quotient were
139 also calculated at the end of the study period (May 2019).

140

141 Galicia has an oceanic climate (a humid sub-tropical climate according to FAO's agro-
142 ecological zoning) with high atmospheric humidity caused by high rainfall and mild
143 temperatures throughout the year (Martínez-Cortizas and Pérez-Alberti, 1999). The
144 climate conditions in Santiago de Compostela during the study period (May/2018–
145 May/2019) are shown in Table 2.

146

147 **2.3. Colour changes**

148

149 Colour was measured on moistened trial areas, according to studies conducted by Prieto
150 et al. (2010b) and Sanmartín et al. (2012). In all areas except the untreated (colonized)
151 reference area, the colour was measured successfully. The measurements could not be
152 made in the colonized area because of the high hydrophobicity of the biofilm
153 (Sanmartín et al., this issue), which prevented it reaching the moisture content of more
154 than 50% required for reproducible measurements (Prieto et al., 2010b).

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The measurements were made with a portable spectrophotometer (Konica Minolta CM-700d) equipped with CM-S100w (SpectraMagic™ NX) software. The working conditions are summarised as follows: medium area view (MAV) 8 mm, illuminant D65, observer 2°, and SCI mode. Measurements were expressed in the CIELAB colour system (CIE, 1986), which represents each colour by means of three parameters: lightness (L*), which varied from 0 (absolute black) to 100 (absolute white), a*(+a*: red and -a*: green) and b* (+b*: yellow and -b*: blue). Of the three CIELAB parameters, only the chromatic parameters, i.e. a* (associated with changes in redness-greenness) and b* (associated with changes in yellowness-blueness), proved relevant for evaluating the efficacy of the treatments. Thus, partial differences in these parameters were determined using the following equations:

$$\Delta a^* = a^*_i - a^*_0$$

$$\Delta b^* = b^*_i - b^*_0$$

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where the subscript i denotes the average value of the parameter in the trial area after one, three, six and twelve months, and the subscript 0 denotes the average value of parameter in the trial area immediately after the treatments. Positive values of Δa^* indicate reddening, and negative values indicate greening. Positive values of Δb^* indicate yellowing, and negative values indicate blueing.

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The number of colour measurements carried out in the trial areas was established following the methodology proposed by Prieto et al. (2010a). Thus, on the first day of the study a total of 125 measurements were made with replacement at different points randomly selected in each trial area. For each measurement, cumulative averages of the three parameters (L*, a*, and b*) representing the colour (CIE, 1986) were plotted against the number of measurements. The stable section of each graph corresponds to the minimum number of measurements required to characterize the value of each parameter accurately and reproducibly, and the largest number obtained was thus considered the minimum number of measurements necessary.

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2.4. *Presence and vitality of organisms determined by PAM fluorometry*

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Chlorophyll-a fluorescence (ChlaF) measurements were made at pre-dawn, to guarantee an ample dark-adaptation time to allow all PSII reaction centres to open, with a multi-wavelength PAM fluorometer (Phyto-PAM) (Heinz Walz GmbH, Effeltrich, Germany) equipped with a fibre optics emitter-detector unit (PHYTO-EDF). In each test area, sixteen readings were made at randomly selected points and acquired at different gains (G) between 15 and 24 and PAR: 16. Before the measurements, each test area was sprayed with distilled water and covered with a black opaque plastic under which measurements were carried out. As in the case of colour measurements, the high hydrophobicity of the biofilm prevented ChlaF measurements being carried out in the

195 untreated (colonized) area. The Fv/Fm ratio, which describes the quantum
196 photochemical efficiency of PSII, was used to check the vitality of the photosynthetic
197 organisms present (Favero-Longo et al., 2017; Pozo-Antonio and Sanmartín, 2018).

198 199 **2.5. Chlorophyll-a extracted and phaeophytization quotient**

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201 At the end of experiment, i.e. twelve months after the treatments, the biomass
202 colonizing the trial areas was collected using swabs moistened with sterile buffer
203 solution (2 mL) and transported to the laboratory in a cool box. The component
204 microorganisms were dispersed in the same buffer by vortexing at 2,000 rpm for 2 min.
205 The resulting cell suspension was centrifuged and resuspended in 2 mL of
206 dimethylsulfoxide (DMSO, Sigma Aldrich) and heated to 65 °C for 1 h, according to
207 Bell and Sommerfeld (1987). The samples were centrifuged for 15 min at 5000g and the
208 supernatant was analysed in a UV-Vis Spectrophotometer (Varian Cary 100). The
209 chlorophyll-a content ($\mu\text{g cm}^{-2}$) and phaeophytization quotient in each trial were
210 calculated using the equations proposed by Wellburn (1994) and Martínez-Abaigar and
211 Núñez-Olivera (1998). All analyses were performed in triplicate.

212 213 **2.6. Statistical analysis**

214
215 The data were subjected to multivariate analysis of variance (MANOVA) and Duncan's
216 post-hoc multiple range test ($p\text{-value} \leq 0.05$), implemented in the SPSS statistical
217 programme (version 22.0).

218 219 **3. Results and discussion**

220
221 In all cases, a single application of the treatment successfully removed the algal biofilm
222 (as assessed by naked eye observation). The biofilm was superficial and was easily
223 removed by the mechanical action that all the treatments entailed. However, slight
224 sanding of the granite surface was noted during application, especially of the four
225 products based on quaternary ammonium salts (Biotin R®, Biotin T®, Preventol RI80®
226 and New Des 50®). This was probably due to the abrasive nature of the products (due
227 to the salt contents) or possibly to the already very sandy surface below the biofilm and
228 on the uncolonized areas (Sanmartín et al., this issue). In this respect, Tirado Hernández
229 et al. (2015) reported damage to the substrate (with disaggregation of the stone material)
230 after application of Biotin T® to decorative elements (ataurique) made from a fine-
231 grained fossiliferous limestone mainly composed of CaCO_3 (calcite), in Medina
232 Azahara (Córdoba, Spain).

233
234 A total of 85 surface colour measurements were finally made in each test area,
235 according to the cumulative average values of the L^* , a^* , and b^* colour parameters
236 (Figure S1). The changes in the green-red colour component (Δa^*) and the blue-yellow
237 colour component (Δb^*) during the study period are shown in Figure 3. The results for

238 the colonized area are not included in the figure as the high hydrophobicity of the
239 biofilm (see Sanmartín et al., this issue) prevented its humidification and accurate
240 recording of the colour measurement in this area (Prieto et al., 2010b), as explained in
241 Section 2.3.

242

243 The Δb^* values appeared to be less useful than the Δa^* values for monitoring the
244 recolonization process (Figure 3). Δb^* varied randomly, while Δa^* displayed well-
245 defined trends, as observed in the dry brushing trial. These findings contrast with
246 observations made during the phototrophic recolonization (March 2009–January 2010)
247 of the south-facing granite facade (exposed to rainfall and where the greening followed
248 pathway of the water run-off) in the Supercomputing Centre of Galicia (CESGA,
249 www.cesga.es), built in 1993 in the university campus within the new part of the city of
250 Santiago de Compostela. In this case, parameter b^* was the most informative for
251 detecting the occurrence of phototrophic colonization (Sanmartín et al., 2012). One
252 possible explanation for this difference is the effect of the colour of the rocky substrate
253 where the colonization occurs. Indeed, previous studies of green algal growth on
254 yellowish-brown coloured lithic substrates, such as the granite masonry of the
255 background walls of the cloister (Figure 2), carried out on limestone (Eyssautier-Chuine
256 et al., 2015) and schists (Sanmartín et al., 2019), showed that Δa^* was the best indicator
257 of algal ‘greening’.

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259 Furthermore, according to colorimetric criteria and considering the visual colour
260 difference threshold or just noticeable difference (JND) of one CIELAB unit, which
261 constitutes the lower limit of perception (Wyszecki and Stiles, 1982), Preventol RI80®
262 and water yielded the best results, and the threshold values of the two chromatic
263 coordinates were not exceeded throughout the study. By contrast, New Des 50® yielded
264 highly visible colour changes (> 3 CIELAB units, the upper limit of rigorous colour
265 tolerance or noticeable change in colour: Wyszecki and Stiles 1982; Sanmartín 2012
266 and references therein), with a significant shift towards red and blue components
267 observed one month after application. These changes are not associated with
268 recolonization during the trial, but with a noticeable change in colour of the granite
269 substrate. Application of didecyldimethylammonium (the active agent of New Des 50®)
270 to pinewood has previously been found to cause significant changes in colour, of more
271 than 12 CIELAB units (Ozgenc and Yildiz, 2014). Likewise, a decrease in Δb^* (shift
272 towards blue) was observed after application of a biocide containing
273 didecyldimethylammonium to wall painting mock-ups painted with red minium, blue
274 smalt, green malachite and carbon black (Becherini et al., 2018). Moreover, Biotin R®
275 was significantly more effective than Biotin T®. In a comparative study of New Des
276 50® and Biotin T® carried out by Dresler et al. (2017) in encapsulation/release systems,
277 Biotin T® yielded a faster, more effective response. However, New Des 50® was more
278 effective than Koretrell© for removing *Verrucaria nigrescens* lichen from dolomite (de
279 los Ríos et al., 2016). In previous studies also on lichen, Biotin R® produced the best
280 results, followed by Preventol RI80® and Biotin T®, with New Des 50® being the least
281 effective biocide treatment of the four tested (Tirado Hernández et al., 2015). Finally, in

282 the present study, treatment with ethanol-water mixture (1:1) and benzalkonium
283 chloride yielded similar results, comparable to those obtained with Biotin T®,
284 especially at the end of the experiment. Overall, the colour data showed that there was
285 no evident recolonization by phototrophic organisms after one year on all the trial
286 cleaning areas. By contrast, in a previous experiment conducted in Santiago de
287 Compostela almost ten years ago, ‘greening’ was perceptible after approximately 6
288 months (after cleaning with a brush and water) on granite walls exposed to rainfall
289 (Sanmartín et al., 2012).

290

291 Figure 4 shows the Fv/Fm values of chlorophyll-a fluorescence. Note that again the data
292 from the colonized area were not included, as the stone could not be humidified (see
293 Section 2.4.). Benzalkonium chloride and Preventol RI80® were the only treatments
294 that did not cause changes exceeding the threshold of 0.10 (below which a
295 photosynthetic organism is considered dead) throughout the study. These results are
296 consistent with those reported by Vannini et al. (2018), who found that Preventol
297 RI80® was effective for removing lichens and caused a rapid physiological alteration.
298 In the areas where dry brushing, mechanical cleaning or washing with water was
299 applied, the values are (logically) above this threshold; after 6 and 12 months a
300 significant increase in activity was observed in the ethanol-water (1:1) mixture, Biotin
301 R®, Biotin T® and New Des 50® treatments. There was also a general increase in
302 activity after 6 months (November 2018) when the high relative humidity, which
303 reached 92% (Table 2), increased the activity of *A. lobatus* cells (Sanmartín et al., this
304 issue). The Fv/Fm values did not exceed 0.4 (threshold in dry brushing trial area) and
305 0.25 (for the other test areas), with the threshold being 0.15 in most cases. These values
306 showed that recolonization of the cleaned areas by phototrophs was relatively low (i.e.
307 barely detectable), as values of between 0.5 and 0.6 are obtained from slightly
308 lichenized algal biofilm on granitic rock (Pozo-Antonio and Sanmartín, 2018) and the
309 theoretical maximum value for green algae is around 0.7 (Young and Beardall, 2003).

310

311 The chlorophyll pigment data (Figure 5) were consistent with the previous colour and
312 chlorophyll-a fluorescence data: Preventol RI80® was the most effective treatment,
313 followed by the ethanol-water (1:1) mixture and benzalkonium chloride, with very
314 similar results (especially in the chlorophyll-a content), and then by Biotin R®, Biotin
315 T®, and finally New Des 50®. ‘Greening’ on granite facades begins to be noticeable
316 from $8.43 + 0.24 \cdot 10^{-3}$ µg extracted chl-a cm⁻² (Sanmartín et al., 2012), In the present
317 study, around 3 µg chl-a cm⁻² was the maximum amount extracted in the dry brushing
318 trial area. Furthermore, according to the phaeophytization quotient (Figure 5), New Des
319 50® was not effective for killing phototrophic organisms, and had the same effect as
320 dry brushing on the degradation of chlorophyll-a.

321

322 Overall, the three types of analysis showed that the different treatments were all
323 effective and that although recolonization occurred in the test areas after one year, it
324 was negligible from a measuring point of view and no biofilm was formed. This may be
325 because the green alga *A. lobatus*, the main species in the original biofilm, generally

326 grows slowly unless exposed to optimal conditions of relative humidity, above 97–98%,
327 although also above 90% (Sanmartín et al., this issue), which only occurred in two
328 months (November and December, when the relative humidity reached 92%) during the
329 year of study (Table 2). Although Preventol RI80® yielded good results in the present
330 study and is widely used (e.g. Vannini et al., 2018; Lazzeri et al., 2018), the above
331 considerations, together with the known side effects of chemical biocides (Kakakhel et
332 al., 2019) (especially those based on quaternary ammonium salts), indicate that this
333 compound should probably not be used in this case. Indeed, removal of the biofilm from
334 the rear walls of the processional cloister of the Monastery of San Martiño Pinario is not
335 recommended, for various reasons (Sanmartín et al., this issue). If it is finally decided
336 that the biofilm must be removed, it should be done by cleaning with water and
337 brushing, as in the present study.

338 **4. Conclusions**

339 Although recolonization of the test areas has been barely detectable, the order of
340 effectiveness of the different treatments was established. Thus, on the basis of the
341 medium-term (one year) results, Preventol RI80® proved to be the most effective
342 treatment, followed by benzalkonium chloride and the ethanol-water (1:1) mixture,
343 which yielded similar results, and then by Biotin R® and Biotin T®. New Des 50® had
344 a deleterious effect, with clearly visible colour changes on the granite substrate, and was
345 the least effective in removing the subaerial biofilm formed by *Apatococcus lobatus*
346 (Chodat) J.B.Petersen (Chlorophyta). On the basis of the results achieved after one year
347 of study and concerns about the potential toxicity of the products tested, we recommend
348 that if the biofilm has to be removed from the cloister wall (for whatever reason), this
349 should be done with water and brushing, as in the present study.

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517 Figure 1. Monastery of San Martiño Pinarío. (A) Main entrance. (B) Central patio of the
518 processional cloister. (C) Map of the cloister: the location of the trial cleaning area is
519 indicated by a green rectangle. (D) West-facing wall, where the trial cleaning areas were
520 located.

521

522 Figure 2. Trial cleaning areas on the west-facing wall of the Processional cloister. The
523 white outer frame delimits an area of ca. 400 cm² used for colour measurements and the
524 white inner frame delimits the area of ca. 100 cm² used for chlorophyll fluorescence
525 measurements. Each of these areas was divided into three smaller, replicate areas: A, B,
526 C in the large area and a, b, c in the small central area.

527

528 Figure 3. Changes in the green-red colour component (Δa^*) and blue-yellow colour
529 component (Δb^*) in the trial areas throughout the study period. Bars represent the
530 means \pm standard errors of three replicate areas. Different letters indicate significant
531 differences between the results achieved in the nine trial areas (including a reference,
532 uncolonized area) for the same time (i.e., at 1, 3, 6 or 12 months), determined by post
533 hoc comparisons (Duncan's test, * $p < 0.05$).

534

535 Figure 4. Values of F_v/F_m , the maximum quantum efficiency of photosystem II (PSII)
536 photochemistry, at 665 nm. Bars represent the means \pm standard errors of three replicate
537 areas. Different letters indicate significant differences between the results achieved in
538 the nine trial areas (including a reference, uncolonized area) for the same time (i.e., at 1,
539 3, 6 or 12 months), determined by post hoc comparisons (Duncan's test, * $p < 0.05$).

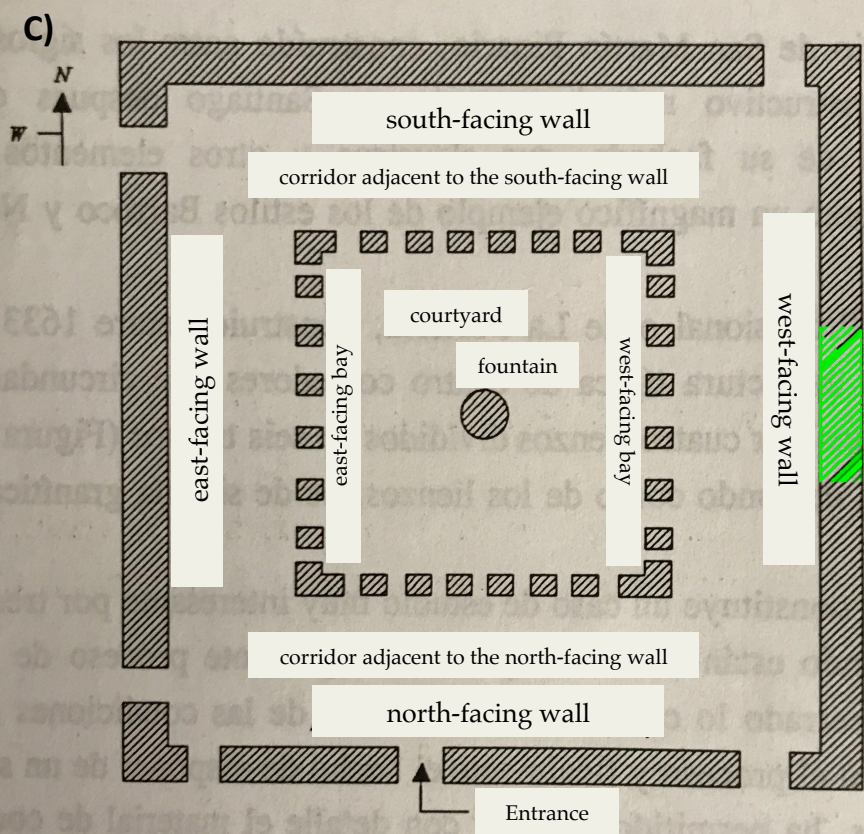
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541 Figure 5. Chlorophyll-a content and phaeophytization quotient, which reflects the ratio
542 of chlorophyll-a to phaeopigments (chlorophyll-a degradation products), in the trial
543 areas at the end of study period (12 months). Bars represent the means \pm standard
544 deviations of three replicates. Different letters indicate significant differences between
545 the results achieved in the nine trial areas (including a reference, uncolonized area)
546 determined by post hoc comparisons (Duncan's test, * $p < 0.05$). The concentration of
547 chlorophyll-a in the colonized area was $2.31 \pm 0.08 \cdot 10^{-2} \mu\text{g cm}^{-2}$ (not shown in the
548 graph).

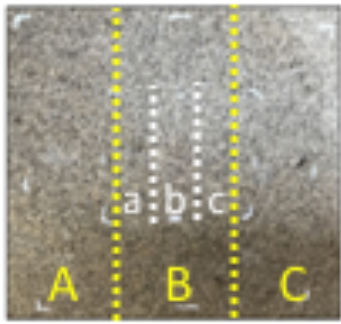
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550 Figure S1. Cumulative average values for parameters L^* , a^* , b^* in relation to the
551 number of measurements, which indicate the minimum number of colour measurements
552 required (Prieto et al., 2010a). Chart of results and example of the graphs used to
553 determine them, with marked segments indicating the minimum number of
554 measurements.

555



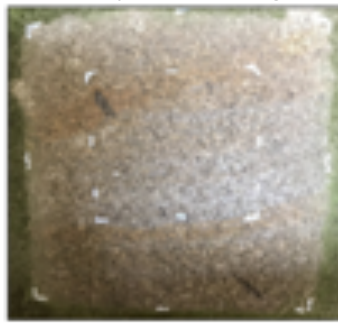
Uncolonized



Colonized



Dry brushing



Water



Ethanol (50%)



Benzalkonium Chloride (3%)



Biotin R (2%)



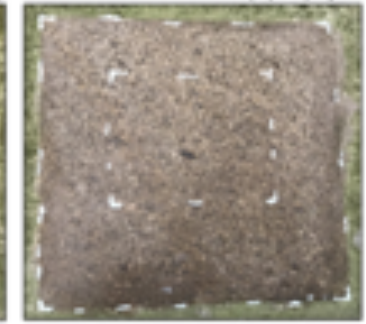
Biotin T (2%)



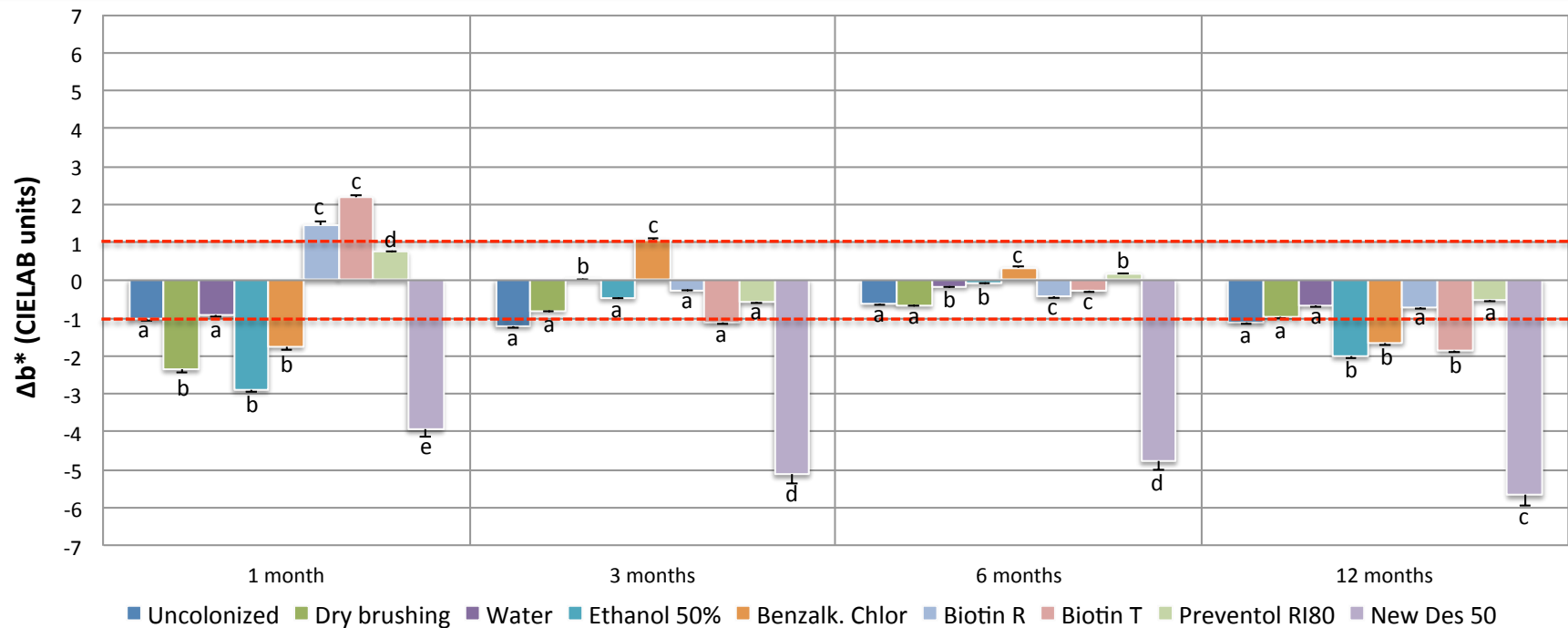
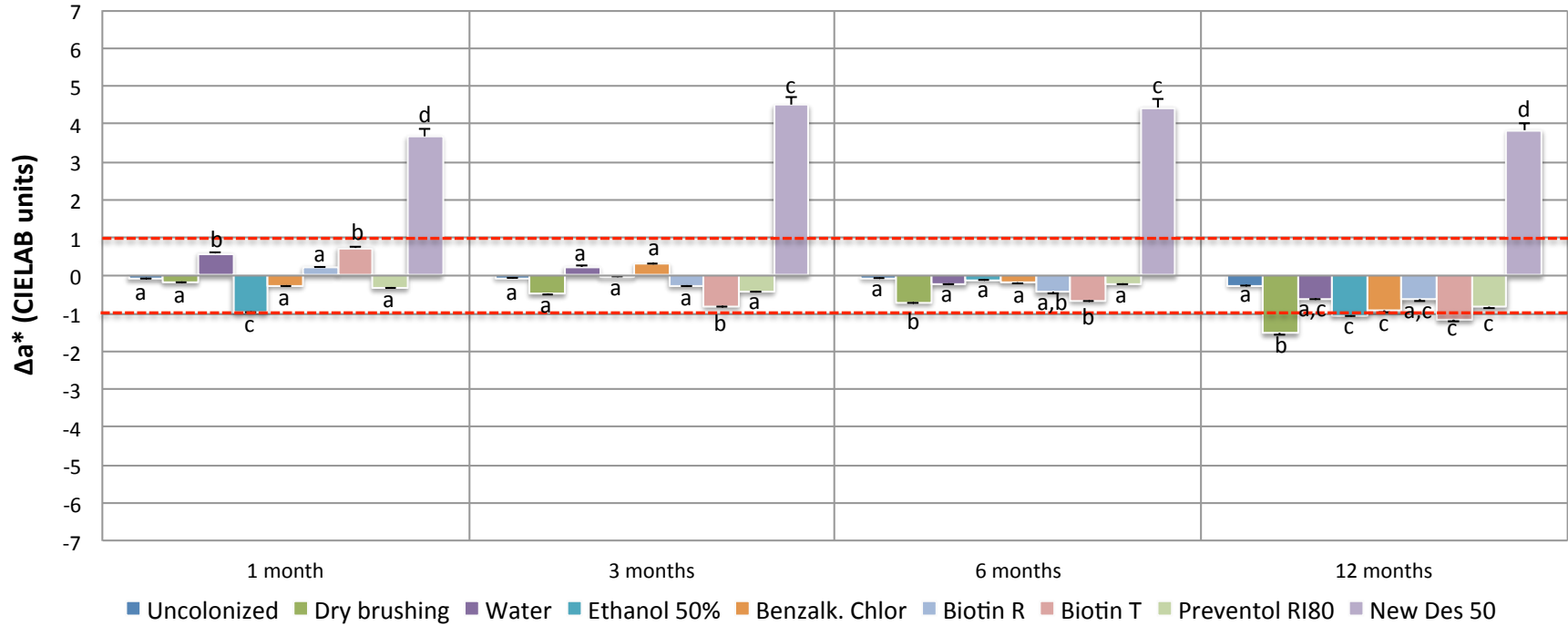
Preventol RI80 (2%)

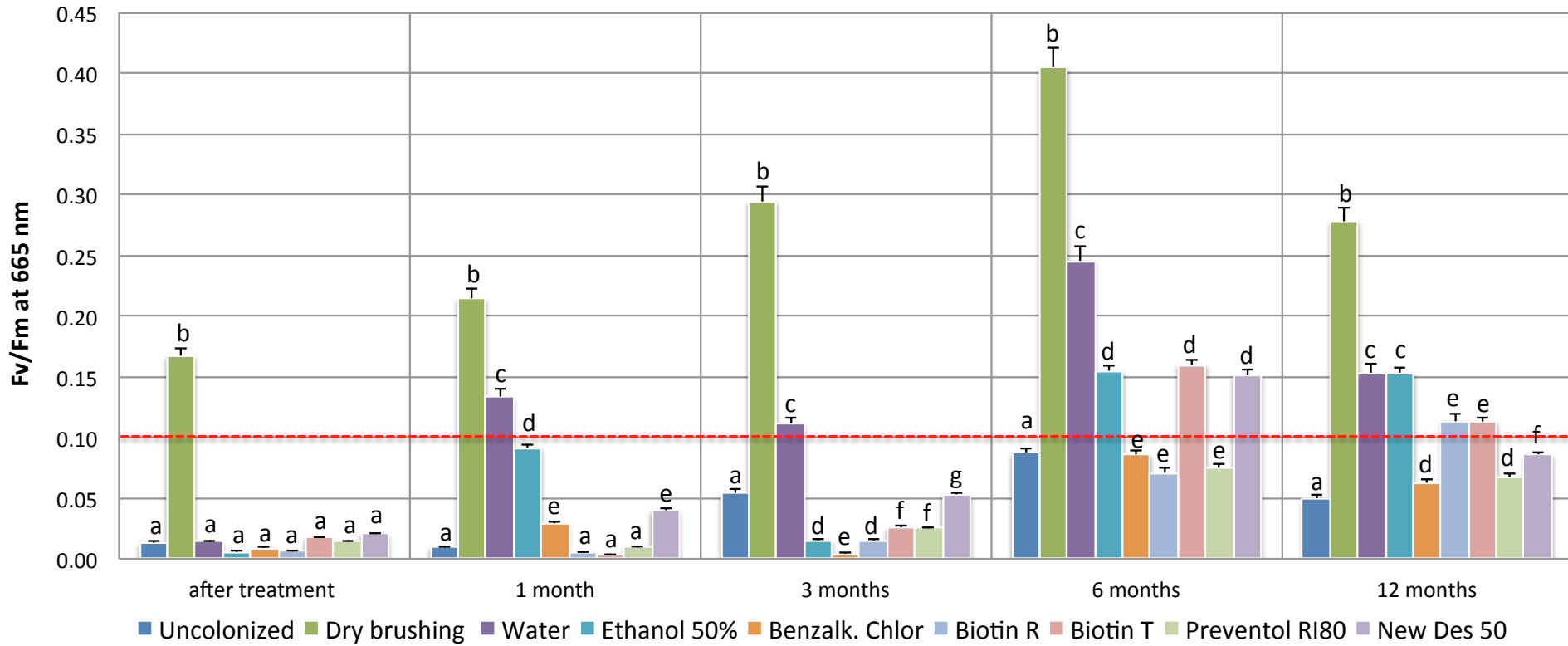


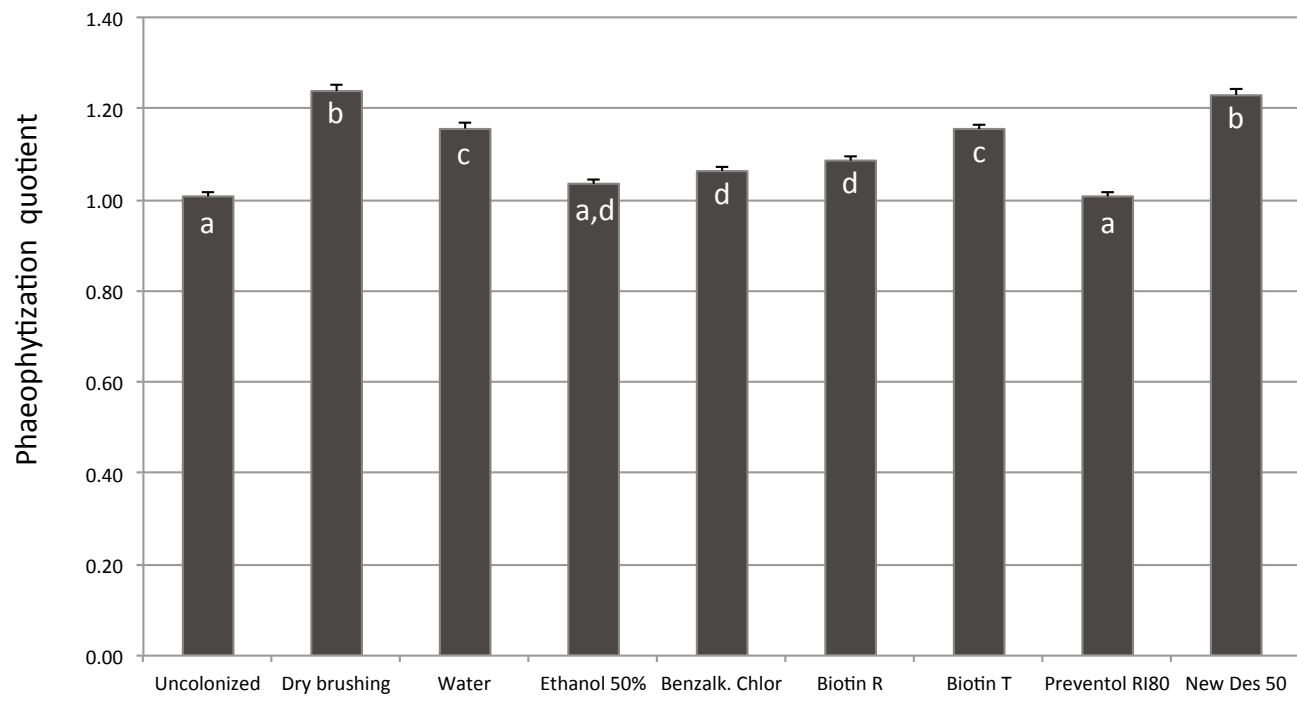
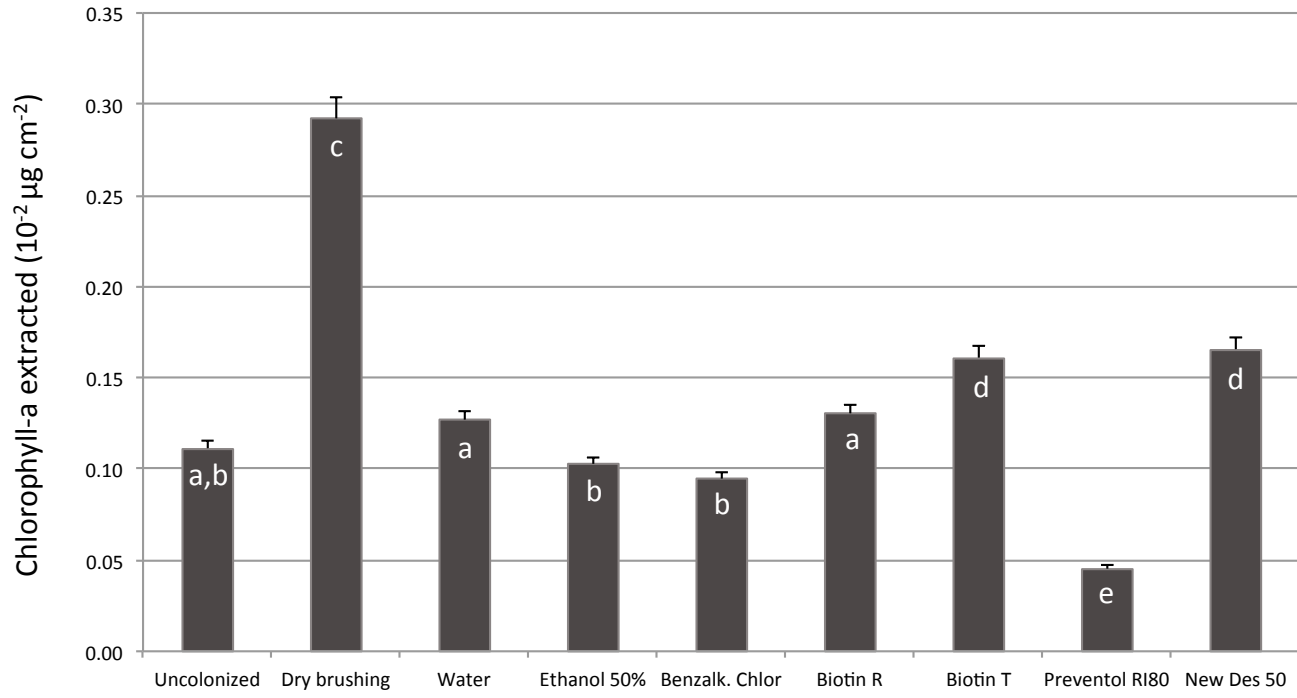
New Des 50 (2%)



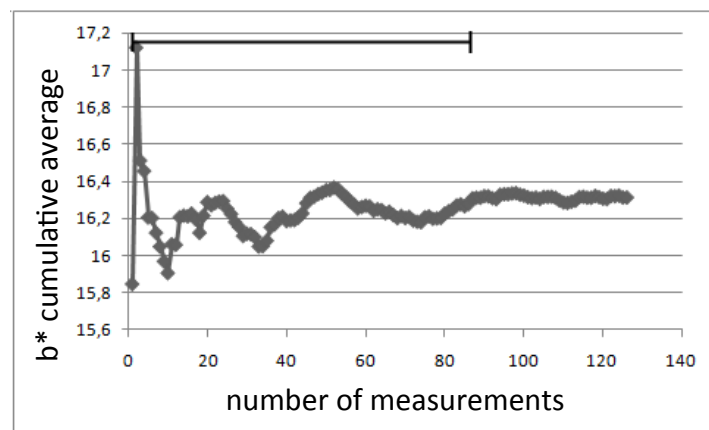
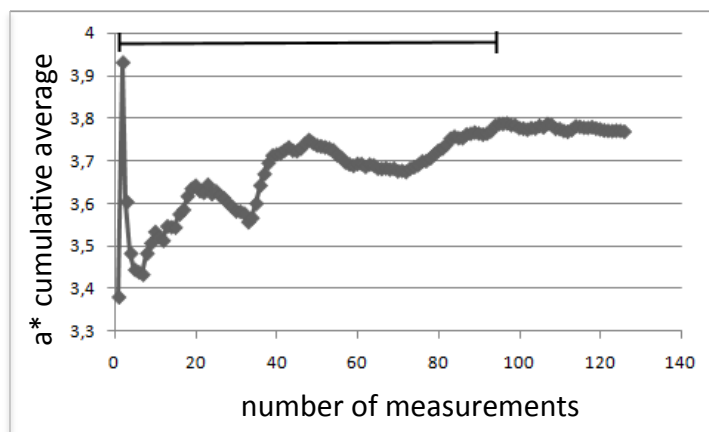
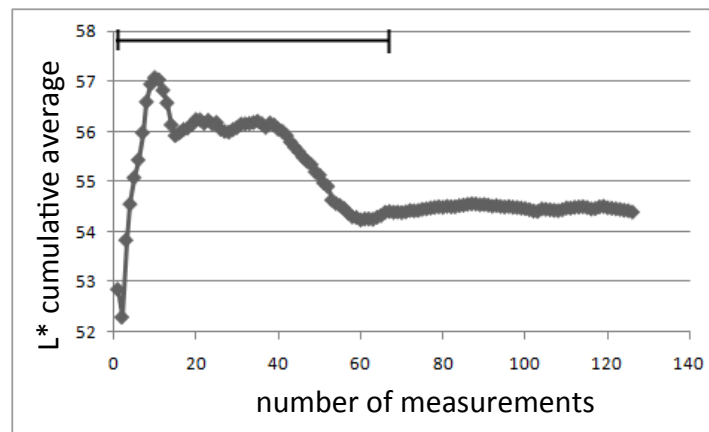
1. Uncolonized
2. Dry Brushing
3. Water
4. Ethanol (50%)
5. Benzalkonium chloride (3%)
6. Colonized
7. Biotin R (2%)
8. Biotin T (2%)
9. Preventol RI80 (2%)
10. New Des 50 (2%)







	L*	a*	b*
Uncolonized	56	70	70
Dry brushing	67	68	85
Water	53	47	64
Ethanol 50%	51	65	48
Benzalk. Chlor	55	57	57
Colonized	85	13	44
Biotin R	54	82	82
Biotin T	85	75	84
Preventol RI80	85	45	28
New Des 50	85	33	60



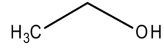
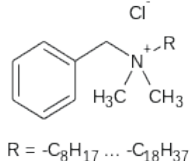
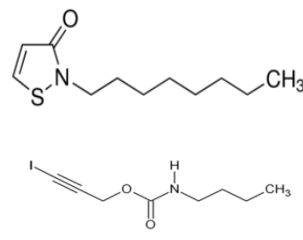
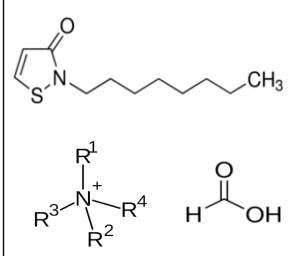
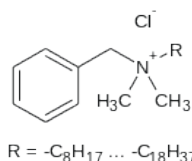
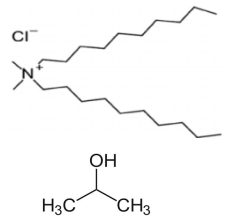
	Ethanol	Benzalkonium chloride	Biotin R®	Biotin T®	Preventol RI80®	New Des 50®
Active Agent	-	Benzalkonium chloride (80%)	N-octyl-isothiazolinone (2,5-10%), Iodopropynyl butylcarbamate (10-25%)	N-octyl-isothiazolinone (2,5-10%), quaternary ammonium salt (25-50%), formic acid (1-2,5%)	Benzalkonium chloride (80%)	N,N-didecyl-N,N-dimethylammonium (50%), isopropanol (20%)
Chemical structure of active agent						
Other compounds (solvents)	-	Ethanol (<10%)	Diethylene glycol monobutyl ether (50%)	Isopropanol (10-25%)	Dipropylene glycol methyl ether (8-12%)	Water (30%)
Highly soluble in	Liquid solvents	Liquid solvents, alcohols	Organic solvents	Alcohols, aromatic hydrocarbons	Liquid solvents, alcohols	Liquid solvents
Biological effects	Destroys the plasma membrane and denatures associated proteins	Inactivates energy-producing enzymes, denatures proteins and destroys the cell membrane	Reacts with intracellular thiols, such as GSH, producing apoptosis. Unknown effect, the toxicity of the iodine probably modifies the permeability of the cell membrane and denatures associated proteins.	Reacts with intracellular thiols, such as GSH, producing apoptosis. Destroys the cell membrane. Inhibits cytochrome C oxidase, producing hypoxia	Inactivates energy-producing enzymes, denatures proteins, and destroys the cell membrane.	Increases membrane permeability, releasing intracellular constituents. Destroys the plasma membrane and denatures associated proteins
Supplier	-	Agar Agar SL	CTS Europe SL	CTS Europe SL	CTS Europe SL	CTS España SL
Price (Euro per Kg) *	-	9.99	45.30	44.65	21.00	20.15

Table 1. Characteristics and properties of the six cleaning-restoration chemical products used in the study. * Up-to-date valid prices in July 2019.

Date	Average air temperature (°C)	Dew point temperature (°C)	Mean relative humidity (%)	Rain (L m ⁻²)	Sunshine duration (h)
May 2018	14.38	10.05	77	28.6	207.5
June 2018	17.12	14.4	86	134.3	164.28
July 2018	19.3	16.04	83	51.5	nd
August 2018	20.64	15.6	75	23.9	260.45
September 2018	19.89	15.03	76	1.4	241.73
October 2018	14.23	9.88	78	152.3	200.73
November 2018	10.97	9.5	92	379.3	83.58
December 2018	10.7	9.33	92	213.6	87.61
January 2019	7.9	5.31	85	150.3	133.66
February 2019	10.16	6.12	79	84.3	158.89
March 2019	10.86	5.71	74	118.1	197.5
April 2019	11.2	7.4	80	197.3	155.54
May 2019	15.03	9.3	72	51	255.12

Table 2. Weather data in Santiago de Compostela city during the study period, from May 2018 to May 2019 (recorded at the Santiago EOAS meteorological station and captured by www.meteogalicia.gal). nd: No data available

Santiago de Compostela, Spain. August 1, 2019

Disclosure of potential conflict of interest

The authors declare that they have no conflicts of interest.

Patricia Sanmartín, on behalf of the authors.

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Medium-term field evaluation of several widely used cleaning-restoration techniques applied to algalphototrophie biofilm formed on a granite-built historical monument

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1 **Abstract**

2 This study evaluated the medium-term performance of some of the most widely used
3 methods ~~of for of~~ cleaning stone; ~~including~~ dry brushing ~~or and~~ washing with water; ~~or~~
4 ~~with an~~ ethanol-water (1:1) ~~mixture~~, benzalkonium chloride; ~~or with and/or the~~
5 commercial products ~~with biocidal properties~~ based on quaternary ammonium salts ~~with~~
6 ~~biocidal properties~~ (Biotin R®, Biotin T®, Preventol RI80®, and New Des 50®). The
7 ability of the ~~different~~ methods to remove ~~an phototrophic-algal~~ biofilm formed on a
8 granite-built historical monument was compared. The biofilm has been shown to affect
9 the appearance, but not the integrity, of the building material. ~~The~~ In the cleaning trials,
10 the presence and vitality of the organisms in colonizing the trial cleaning areas substrate
11 were determined, immediately after treatment and after one, three, six and twelve
12 months, by ~~colour spectrophotometry spectrophotometric colour~~ and chlorophyll-a
13 fluorescence (ChlaF) analysis. The quantity and physiological state of ~~the~~ biofilm-
14 forming phototrophs in the test areas were also determined one year after the treatments.
15 ~~Although a manifest recolonization onf the ttrial est areas has not occurred was barely~~
16 ~~noticeable was negligible after all of the treatments,~~ Preventol RI80® proved to be the
17 most effective ~~treatment~~, followed by benzalkonium chloride and ~~the~~ ethanol-water
18 (1:1) ~~mixture~~, which yielded similar results. New Des 50® caused clearly visible colour
19 changes on the granite wall, and the biocidal efficacy was poor. Biotin R® performed
20 better than Biotin T®, which yielded similar results to New Des 50® regarding
21 recolonization. ~~Based on~~ Considering the overall results and also and the potential
22 sustainability toxicity of the tested products tested, cleaning with water is recommended
23 if and if cleaning is when required.

24 **Keywords (maximum of 6):** cleaning efficacy; commercial biocides; monitoring
25 techniques; phototrophic colonization; recolonization; tertiary bioreceptivity.

26

27 1. Introduction

28 Removal of colonizing organisms from the surface of historical monuments and
29 structures is controversial, as the ~~deteriorative or negligible (or even protective)~~ effects
30 of the colonizing communities ~~(which may be deteriorative, negligible or even~~
31 ~~protective)~~ must be carefully considered and weighed against aesthetic and site
32 management factors (Pinna, 2014; Bartoli et al., 2014; Salvadori and Casanova-
33 Municchia, 2016; Caneva et al., 2018). The community biodiversity and related factors
34 must also be taken into account (Cicinelli et al., 2018, 2019), and the reality is that
35 cleaning and conserving historical stone buildings is generally a very complicated
36 procedure. Indeed, the cleaning of heritage sites involves a huge financial outlay (see
37 e.g. Europanostr.org). According to the Santiago de Compostela (Spain) city council,
38 the cost of removing subaerial biofilms (SABs) from the main facade (Obradoiro) of the
39 Pazo de Raxoi (city council buildings) in 2016 amounted to €318,000. As this is a
40 routine task carried out by restorers, conservators and architects, detailed study of the
41 effectiveness and impact of cleaning treatments is very important in order to establish
42 strategies of controlling or preventing biological colonization on buildings.

43
44 The efficacy of cleaning treatments depends on a number of factors, such as the target
45 species, environmental conditions, mechanical removal strength and product application
46 (see e.g. Favero-Longo et al., 2017; Kakakhel and Wu et al. 2019). Product selection is
47 usually based on the experience of the restorers and the availability of commercial
48 formulations, supported by empirical evaluations that in some cases report negative side
49 effects, such as in the famous Lascaux Cave (Kakakhel and Wu et al. 2019).

50
51 On-site monitoring of treatment efficacy is an essential part of the intervention. ~~To~~
52 ~~instead of for~~ For monitoring the phototrophic components of subaerial biofilms
53 (SABs), ~~non~~Non-destructive analysis to determine the biomass and physiological state
54 of phototrophs is ideal ~~for monitoring purposes. and is preferable to determining the~~
55 ~~phototrophic components of subaerial biofilms (SABs) by destructive analysis.~~ In this
56 regard, colour measurement expressed in the CIELAB space (CIE Publication 15-2,
57 1986) has been firmly established as a proxy indicator for early quantification of
58 phototrophic subaerial biofilm formation on granite stone (e.g. Sanmartín 2012 and
59 references therein; Sanmartín et al., 2012; Pozo-Antonio et al., 2017). The technique is
60 also suitable for determining the response of phototrophic microorganisms to biocidal
61 strategies, as L* and a* CIELAB parameters are closely related to physiological
62 parameters such as photosynthetic pigments (chlorophyll-a, phaeopigments) and ATP
63 content (Sanmartín et al., 2011, 2015). Likewise, the chlorophyll-a fluorescence (ChlaF)
64 parameter Fv/Fm (maximum quantum efficiency of PSII) has been reported to be
65 suitable for ascertaining the vitality of algal remains of subaerial biofilms on granite
66 (Pozo-Antonio and Sanmartín, 2018).

67
68 In this research, we evaluated the medium-term (one year) efficacy of some of the ~~stone~~
69 ~~cleaning currently methods currently most widely used by conservators and~~
70 ~~restorers methods for instead of cleaning stone~~, particularly ~~for cleaning~~ granite-built

71 heritage, ~~by conservators and restorers~~. The treatments, which include dry brushing,
72 washing with water, ethanol-water (1:1), benzalkonium chloride or with commercial
73 products based on quaternary ammonium salts with biocidal properties ~~based on~~
74 quaternary ammonium salts (Biotin R®, Biotin T®, Preventol® RI80, and New Des®
75 50) were tested on a ~~subaerial biofilm~~ highly hydrophobic subaerial biofilm, formed by
76 *Apatococcus lobatus* (Chodat) J.B.Petersen (Chlorophyta), ~~colonizing on~~ the
77 processional cloister of the Monastery of San Martiño Pinario (Sanmartín et al., this
78 issue). ~~The~~ In the cleaning trials, the presence and vitality of the organisms ~~in~~
79 colonizing the substrate the trial cleaning areas were ~~analyzed determined~~, immediately
80 after cleaning and after one, three, six and twelve months, by colour
81 spectrophotometry and modulated chlorophyll-a fluorescence
82 measurements analysis spectrophotometric colour and chlorophyll-a fluorescence
83 (ChlaF) analysis. The quantity and physiological state of biofilm-forming phototrophs
84 on the ~~trial cleaning test~~ areas were also analysed one year after the treatments.

85 2. Materials and methods

86 2.1. Study area

87
88 The target building under consideration is ~~located~~ adjacent to the cathedral in the
89 historical centre of Santiago de Compostela (UNESCO World Heritage City since 1985,
90 capital of Galicia, NW Spain). The Monastery of San Martiño Pinario (UTM 29T X
91 537220, Y 4747793; Datum ETRS89, Figure 1), which was built between the 9th and
92 18th centuries, has a distinguished architecture ranging from baroque to neoclassic, and
93 ~~it~~ is the most important constructive complex in the city after the cathedral. ~~It has~~ also
94 been marked as considered the most valuable baroque building in Galicia. At the end of
95 2019, the procedure for recognizing -declare the Monastery as an Asset of Cultural
96 Interest for the year 2021 (Xacobeo, Holy Year) has begun was initiated at the end of
97 2019.

98
99 The monastery's processional cloister of granite stonework, built between 1633 and
100 1747, has a traditional structure of sober, classic lines, ~~with including~~ four bays
101 surrounding a courtyard with a fountain in the centre (Figure 1). It has two floors, ~~- -~~ a
102 high cloister and a lower cloister, ~~- -~~ joined by paired columns of monumental Tuscan
103 order, each set on a high plinth. The first floor, or lower cloister, is articulated by means
104 of half-point arches, while the upper cloister has vaulted flat balconies, with pairs of
105 large Doric columns in the intermediate spaces. The whole building is enclosed by ~~the a~~
106 bell tower. Although the project is attributed to Bartolomé Fernández Lechuga, ~~it also~~
107 involved Peña del Toro and Miguel de Romay were also involved, the latter as designer
108 of the bell tower.

109
110 Further information about the granite masonry of the background walls of the cloister,
111 as well as the joint mortar, is reported in Sanmartín et al. (this issue) and references
112 therein. Where a comprehensive explanation regarding the colonization pattern

113 outlined by the subaerial biofilm growth through the background walls is also given in
114 the aforementioned paper.

115 116 2.2. *Cleaning treatments*

117
118 Eight adjacent areas on the west-facing background wall of the processional cloister of
119 the Monastery of San Martiño Pinario (biofilm cover) (Figure 2), all with the same level
120 (or degree of presence) of biological colonization, were selected according to previous
121 studies by Pozo-Antonio et al. (2017) and Pinna et al. (2018). In each trial area, one of
122 the 8 treatments was applied with a brush to the subaerial biofilm formed by
123 *Apatococcus lobatus* (Chodat) J.B.Petersen (Chlorophyta) (Sanmartín et al., this issue).
124 The biofilm was removed following the method described by Pozo-Antonio and
125 Sanmartín (2018). One untreated (colonized) area and one ~~uncolonised~~ uncolonized area
126 adjacent to the treated areas were ~~also analyzed~~ analysed for reference purposes (Figure
127 2).

128
129 The ~~following 8~~ treatments were ~~applied as follows~~: dry brushing; washing with
130 water; ~~washing with an~~ ethanol-water mixture (1:1), ~~i.e. ethanol at 50% (v/v in~~
131 distilled water), as in previous studies on granitic rocks (Pozo et al., 2013; Pozo-
132 Antonio et al., 2017); ~~washing with~~ benzalkonium chloride; ~~and washing or~~ with
133 Biotin R®, Biotin T®, Preventol RI80® or New Des 50® ~~the following~~ (commercial
134 products based on quaternary ammonium salts with biocidal properties ~~based on~~
135 quaternary ammonium salts); ~~Biotin R®, Biotin T®, Preventol RI80® and New Des~~
136 50®. A d Detailed descriptions of the six chemical products ~~is are~~ provided in Table 1.

137
138 Each ~~treated area to which with a single application of each treatment was applied~~
139 (single application) measured about 20×20 cm. At the end of the experiment, colour
140 measurements were made (section 2.3) and chlorophyll pigments were extracted
141 (section 2.5) in the whole area, which was divided into 3 replicate areas, each of ca. 120
142 cm². Due to the small size (4 mm in diameter) of the measuring head of the Pulse-
143 Amplitude-Modulation (PAM) chlorophyll fluorometer, a smaller, central area, ~~(of~~
144 about 10×10 cm,) in each ~~trial test~~ area was also divided into 3 replicate areas, ~~(each of~~
145 ca. 30 cm²); for chlorophyll fluorescence measurement (section 2.4).

146
147 Recolonization was monitored in each trial area throughout a period of 12 months
148 (May/2018–May/2019), i.e. immediately after treatments (May 2018) and after one
149 month (June 2018), three months (August 2018), six months (November 2018) and
150 twelve months (May 2019) by ~~colour spectrophotometry~~ spectrophotometric colour
151 and modulated chlorophyll-a fluorescence measurements analysis ~~chlorophyll-a~~
152 fluorescence analysis. The chlorophyll-a content and phaeophytization quotient were
153 also calculated at the end of the study period (May 2019).

154
155 Galicia has an oceanic climate (a humid sub-tropical climate according to FAO's agro-
156 ecological zoning) with high atmospheric humidity caused by high rainfall and mild

157 temperatures throughout the year (Martínez-Cortizas and Pérez-Alberti, 1999). The
158 weather-climate conditions in Santiago de Compostela city during the study period
159 (May/2018–May/2019) are shown in Table 2.

161 2.3. Colour changes

162
163 Colour was measured on moistened trial areas, according to studies conducted by Prieto
164 et al. (2010b) and Sanmartín et al. (2012). In all areas except the untreated (colonized)
165 reference area untreated (colonized), the colour was measured successfully. The reason
166 for not being able to measurements could not be made in the colonized area was
167 itsbecause of the high hydrophobicity of the biofilm (Sanmartín et al., this issue), which
168 did not allow to prevented it reaching the biofilm to reach a moisture content of more
169 than 50% required to performfor reproducible measurements (Prieto et al., 2010b).

170
171 The measurements were made with a portable spectrophotometer (Konica Minolta CM-
172 700d) equipped with CM-S100w (SpectraMagic™ NX) software. The working
173 conditions are summarised as follows: medium area view (MAV) 8 mm, illuminant
174 D65, observer 2°, and SCI mode. Measurements were expressed in the CIELAB colour
175 system (CIE, 1986), which represents each colour by means of three parameters:
176 lightness (L*), which varied from 0 (absolute black) to 100 (absolute white), a*(+a*:
177 red and -a*: green) and b* (+b*: yellow and -b*: blue). Of the three CIELAB
178 parameters, only the chromatic onesparameters, i.e. a* (associated with changes in
179 redness-greenness) and b* (associated with changes in yellowness-blueness), proved
180 relevant tofor evaluateing the treatments' efficacy and effectivenessof the treatments.
181 Thus, pL*, lightness; a*, green-red component; and b*, blue-yellow component. Partial
182 differences in ehromatic these parameters (a* and b*) were determined using the
183 following equations:

$$184 \Delta a^* = a^*_i - a^*_0$$

$$\Delta b^* = b^*_i - b^*_0$$

185 where the subscript i denotes the average value of the parameter in the trial area after
186 one, three, six and twelve months, and the subscript 0 denotes the average value of
187 parameter in the trial area immediately after the treatments. Positive values of Δa^*
188 indicate reddening, and negative values indicate greening. Positive values of Δb^*
189 indicate yellowing, and negative values indicate blueing.

190
191 The number of colour measurements carried out in the trial areas was established
192 following the methodology proposed by Prieto et al. (2010a). Thus, on the first day of
193 the study a total of 125 measurements were made with replacement at random-different
194 points randomly selected in each trial area. For each measurement, cumulative averages
195 of the three parameters (L*, a*, and b*) representing the colour (CIE, 1986) were
196 plotted against the number of measurements. The stable section of each graph
197 corresponds to the minimum number of measurements required to characterize the value

198 of each parameter accurately and reproducibly, and the largest number obtained was
199 thus considered the minimum number of measurements necessary.

201 **2.4. Presence and vitality of organisms determined by PAM fluorometry**

202
203 Chlorophyll-a fluorescence (Chl_aF) measurements were made at pre-dawn, to
204 ensure guarantee an ample dark-adaptation time sufficientample-fo to allow all PSII
205 reaction centres to open, with a multi-wavelength PAM fluorometer (Phyto-PAM)
206 (Heinz Walz GmbH, Effeltrich, Germany) equipped with a fibre optics emitter-detector
207 unit (PHYTO-EDF). In each trialtest area, sSixteen readings were made at randomly
208 selected points and acquired at different gains (G) between 15 and 24 and PAR: 16.
209 Before the measurements, each test trial-area was sprayed with distilled water and
210 covered with a black opaque plastic under which measurements were carried out.
211 Furthermore, aAs in the case of colour measurements, the high hydrophobicity of the
212 biofilm prevented Chl_aF the inability to moisturizemeasurements being carried out in
213 the untreated (colonized) area prevented performing the measurements there. The
214 F_v/F_m ratio, which describes the quantum photochemical efficiency of PSII, was used
215 to check the vitality of the photosynthetic organisms present (Favero-Longo et al., 2017;
216 Pozo-Antonio and Sanmartín, 2018).

218 **2.5. Chlorophyll-a extracted and phaeophytization quotient**

219
220 At the end of experiment, i.e. twelve months after the treatments, the biomass
221 colonizing the trial areas was collected by-using swabs moistened with sterile buffer
222 solution (2 mL) and transported to the laboratory in a cool box. The component
223 microorganisms were dispersed in the same buffer by vortexing at 2,000 rpm for 2 min.
224 The resulting cell suspension was centrifuged and resuspended in 2 mL of
225 dimethylsulfoxide (DMSO, Sigma Aldrich) and heated to 65 °C for 1 h, according to
226 Bell and Sommerfeld (1987). The samples were centrifuged for 15 min at 5000g and the
227 supernatant was analysed in a UV-Vis Spectrophotometer (Varian Cary 100). The
228 chlorophyll-a content (µg cm⁻²) and phaeophytization quotient in each trial were
229 calculated using the equations proposed by Wellburn (1994) and Martínez-Abaigar and
230 Núñez-Olivera (1998). All analyses were performed in triplicate.

232 **2.6. Statistical analysis**

233
234 The data were subjected to multivariate analysis of variance (MANOVA) and Duncan's
235 post-hoc multiple range test (p-value ≤ 0.05), implemented in the SPSS statistical
236 programme (version 22.0).

238 **3. Results and discussion**

240 ~~In all cases~~The removal of algal biofilm with a, a single application of the treatment of
241 ~~treatment was successfully~~ removed the algal biofilm (as assessed by ~~to the~~ naked eye
242 ~~observation~~) ~~for all cases~~. The biofilm was superficial and was easily removed very
243 ~~easily~~ by the mechanical action that all the treatments entailed. ~~On the other~~
244 ~~hand~~However, a slight sanding of the granite surface ~~when applying the treatment~~ was
245 ~~noted during application~~, especially of ~~with the~~ four products based on quaternary
246 ammonium salts (Biotin R®, Biotin T®, Preventol RI80®, and New Des 50®). This
247 ~~was probably associated with~~due to the abrasive nature effect of the products~~t~~ (due to
248 ~~the salt contents~~) that ~~allocates that texture when is applied due probably to its content~~
249 ~~in salts, or maybe, possibly~~ to the already very sandy surface ~~underneath~~below the
250 biofilm as well as ~~and~~ on the uncolonized areas (Sanmartín et al., this issue). In this
251 ~~sense~~respect, Tirado Hernández et al. (2015) using the same four products based
252 quaternary ammonium salts Tirado Hernández et al. (2015) reported ~~on the damage to~~
253 the substrate (with ~~the~~ disaggregation of the stone material) ~~when~~after application of
254 Biotin T® was applied to ~~pieces of atauriques~~ decorative elements (ataurique) made
255 ~~off from a fine-grained fossiliferous limestone,~~ mainly composed mostly of CaCO₃
256 (calcite), in Medina Azahara (Córdoba, Spain).

257
258 A total of 85 surface colour measurements were finally made in each ~~trial~~ test area,
259 according to ~~the the results of the~~ cumulative average values ~~for of~~ the L*, a*, and b*
260 colour parameters (Figure S1). The changes in the green-red colour component (Δa^*)
261 and the blue-yellow colour component (Δb^*) during the study ~~period~~ are shown in
262 Figure 3. The results for the colonized area are not included in the figure as the high
263 hydrophobicity of the biofilm (~~see~~ Sanmartín et al., this issue) prevented ~~its~~
264 humidification and accurate recording of the colour measurement in this area (Prieto et
265 al., 2010b), ~~as explained in Section 2.3~~.

266
267 ~~According the Figure 3, a~~Analysis of the Δb^* values was appeared to be less useful than
268 ~~of the~~ Δa^* values for monitoring the recolonization process (Figure 3). Δb^* showed
269 ~~variations~~varied more erratic and blurred trends, randomly, while Δa^*
270 ~~presented~~displayed well-defined trends, such as ~~that~~ observed in the dry brushing trial.
271 ~~This~~These findings is ~~contrary to~~contrast with the ~~previous results~~ observations made
272 ~~during~~ obtained in the phototrophic recolonization ~~process~~ (March /2009–January
273 /2010) of the south-facing granite facade, (~~accessible~~exposed to the rainfall and
274 ~~with~~where the greening developed ~~following~~followed pathway of the ~~path of~~ water run-
275 ~~off~~) ~~of~~in the Supercomputing Centre of Galicia (CESGA, www.cesga.es), built in 1993
276 in the university campus ~~inside~~ within the new part of the city of Santiago de
277 Compostela. ~~where~~In this case, parameter b* was the most informative for detecting
278 the occurrence of phototrophic colonization (Sanmartín et al., 2012). One possible
279 ~~explanation for this fact~~ difference is the effect of the coloration~~ur~~ of the rocky substrate
280 ~~on~~where ~~which~~the colonization occurs. Indeed, previous ~~works~~ studies of green algal
281 growth on yellowish-brown color~~ou~~red lithic substrates, such as the granite masonry of
282 the background walls of the cloister (Figure 2), carried out on limestone (Eyssautier-

283 Chuine et al., 2015) and schists (Sanmartín et al., 2019), had already shown showed that
284 Δa^* as was the best indicator monitoring of algal 'greening'.

285
286 Furthermore, a According to colorimetric criteria and considering the visual colour
287 difference threshold or just noticeable difference (JND) of one CIELAB unit, which
288 constitutes the lower limit of perception (Wyszecki and Stiles, 1982), Preventol RI80®
289 and water yielded the best results, and not exceeding the threshold values in either of the
290 two chromatic coordinates were not exceeded throughout the study. By contrast, New
291 Des 50® yielded highly visible colour changes (> 3 CIELAB units, the upper limit of
292 rigorous colour tolerance or noticeable change in colour, ~~Wyszecki and Stiles 1982;~~
293 Sanmartín 2012 and references therein), with a significant shift towards red and blue
294 components observed, one month after application. These changes are not associated
295 with recolonization during the trial, but with a noticeable ~~color~~ change in colour of the
296 granite substrate. Application of didecyldimethylammonium (the active agent ~~in of~~ New
297 Des 50®) (didecyldimethylammonium) to pinewood has previously been found to
298 cause significant changes in colour, above of more than 12 CIELAB units (Ozgenç and
299 Yildiz, 2014). Likewise, a decrease of Δb^* (shift towards blue) was observed after
300 application of a biocide containing didecyldimethylammonium on to wall painting
301 mock-ups painted with red orange-minium, blue smalt, green malachite and carbon
302 black, after applying a biocide which also contained didecyldimethylammonium was
303 reported by (Becherini et al., 2018). Moreover, Biotin R® was significantly more
304 effective than Biotin T®. In a comparative study of New Des 50® and Biotin T®
305 carried out by Dresler et al. (2017) in encapsulation/release systems, Biotin T® yielded
306 a faster, and more effective response. However, New Des 50® was more effective than
307 Koretrell© for the removal of removing *Verrucaria nigrescens* lichen from dolomite (de
308 los Ríos et al., 2016). In previous studies also on lichen, Biotin R® showed produced the
309 best results, followed by Preventol RI80® and Biotin T®, having proven to be with New
310 Des 50® being the least effective biocide treatment of the four tested (Tirado
311 Hernández et al., 2015). Finally, in the present study, treatments with ethanol-water
312 mixture (1:1) and benzalkonium chloride, in the present study, yielded similar results,
313 especially at the end of the experiment relative to the control water treatment and
314 according to the colour results, the treatments that increased the tertiary bioreceptivity
315 of the granite substrate were New Des 50®, ethanol-water (1:1), benzalkonium chloride,
316 and Biotin T®, in that order.

317 omparable to those obtained with Biotin T®, especially at the end of the experiment.
318 Overall, from the colour data can be asserted that there has not been showed that there
319 was no evident recolonization by occurrence of phototrophic organisms after one year
320 on all the trial cleaning areas. Previously, b By contrast, in another a previous experiment
321 conducted in Santiago de Compostela almost ten years ago, 'greening' was perceptible
322 after approximately 6 months (since after cleaning with a brush and water and brush) on
323 granite walls accessible exposed to the rainfall granite walls (Sanmartín et al., 2012).

324
325 Figure 4 shows the Fv/Fm values of chlorophyll-a fluorescence. Note that again the data
326 from the colonized area were not included, as the area-stone could not be humidified

327 (see Section 2.4). Benzalkonium chloride and Preventol RI80® ~~are~~ were the only
328 treatments that did not cause changes exceeding the threshold of 0.10 (below which a
329 photosynthetic organism is considered dead) throughout the study. These results are
330 consistent with those ~~reported by~~ Vannini et al. (2018), who found that Preventol
331 RI80® was effective for removing lichens, ~~with a~~ and caused a rapid physiological
332 alteration ~~response~~. In the areas where dry brushing, mechanical cleaning or washing
333 with water was applied, the values are (logically) above this threshold; after 6 and 12
334 months a significant increase in activity was observed in the ethanol-water (1:1)
335 ~~mixture~~, Biotin R®, Biotin T® and New Des 50® treatments. There was also a general
336 increase in activity after 6 months (November 2018) when the high ~~environmental~~
337 ~~relative~~ humidity, which reached 92% (Table 2), increased the activity of *A. lobatus*
338 cells (Sanmartín et al., this issue). ~~The Fv/Fm values did not exceeded 0.4 (threshold~~
339 ~~in dry brushing trial area) and 0.25 (for the rest of trial other test areas), with being the~~
340 ~~threshold being of 0.15 in most cases. It proved~~ These values showed that recolonization
341 ~~of the cleaned areas by phototrophs was relatively lownegligible (i.e. barely~~
342 ~~detectablewithout biofilm formation) not recolonized, as values of between 0.5 and 0.6~~
343 ~~are obtained from slightly lichenized algal biofilm on granitic rock (Pozo-Antonio and~~
344 ~~Sanmartín, 2018) and the theoretical maximum value for green algae is around 0.7~~
345 ~~(Young and Beardall, 2003).~~

346
347
348
349 ~~€~~ The chlorophyll pigment data (Figure 5) were consistent with the previous colour and
350 chlorophyll-a fluorescence data, ~~with: the order of effectiveness led by~~ Preventol
351 RI80® ~~was the most effective treatment,~~ followed by ~~the~~ ethanol-water (1:1) ~~mixture~~
352 and benzalkonium chloride, with very similar results (especially in the chlorophyll-a
353 content), ~~and then by~~ Biotin R®, Biotin T®, and finally New Des 50®. ~~Sanmartín et al.~~
354 ~~(2012) ‘gGreening’ on a granite facades begins to be noticeable from $8.43 + 0.24 \cdot 10^{-3}$~~
355 ~~μg extracted chl-a cm^{-2} (Sanmartín et al., 2012),- In the , being in the present study,~~
356 ~~barely around $3 \mu\text{g}$ extracted chl-a cm^{-2} was the the maximum amountvalueamount~~
357 ~~extracted, which corresponded to in the dry brushing trial area. Furthermore, according~~
358 ~~to the phaeophytization quotient (Figure 5), New Des 50® was not effective for the~~
359 ~~death ofkilling phototrophic organisms, beingand had the sameits effect as dry brushing~~
360 ~~on the degradation of chlorophyll-a significantly equal to that of dry brush.~~

361
362 Overall, the three types of analyseis ~~confirm~~ showed the same order of effectiveness
363 ~~between~~ that the different treatments were all effective and that although recolonization
364 ~~occurred after a year in any of the trialest areas~~ after one year, it was negligible from a
365 ~~measuring point of view~~ and no biofilm was formed~~has been manifest~~. This ~~e-latter~~ may
366 ~~could be due to~~ because the green alga *A. lobatus*, the main species ~~colonizer in the~~
367 ~~wall in the original biofilm, is a species of green alga with generally low growthgrows~~
368 ~~slowly unless and that increases its growth to exposed to the optimum inoptimal~~
369 ~~conditions of relative humidity (RH), above 97–98%, andalthough also above at lesser~~
370 ~~extent 90% (Sanmartín et al., this issue), which has only been fulfilledonly occurred in~~

371 ~~two months (November and December, when the relative humidity reached WITH A~~
372 ~~RH OF 92%) of during the year of study (Table 2). Taking that into consideration, a in~~
373 ~~the light of the side effects of chemical biocides (Kakakhel et al., 2019), especially~~
374 ~~those based on quaternary ammonium salts such as- Although Preventol RI80®, yielded~~
375 ~~good results despite its good results in the present workstudy and its high application is~~
376 ~~widely used (e.g.: Vannini et al., 2018; Lazzeri et al., 2018), ; t-the- above~~
377 ~~considerations, together with the known side effects of chemical biocides (Kakakhel et~~
378 ~~al., 2019) (especially those based on quaternary ammonium salts), indicate that this~~
379 ~~compound should probably not be used in this case. Indeed, removal of the biofilm from~~
380 ~~the rear walls of the processional cloister of the Monastery of San Martiño Pinario is not~~
381 ~~recommended, for various reasons (Sanmartín et al., this issue). Ifrecommendation if it~~
382 ~~is finally decided that the biofilm must-must be removed-of the background walls of the~~
383 ~~processional cloister of the Monastery of San Martiño Pinario, which was not be~~
384 ~~recommended (Sanmartín et al., this issue) is their should be done by cleaning with~~
385 ~~water and brushing, in the way it has been as done in thie presents study.~~

386 4. Conclusions

387 ~~Although a manifest recolonization on the trial areas has-of the test areas has been~~
388 ~~barely detectable apparently happened, anthe order of effectiveness of the different~~
389 ~~treatments wasean be established between the treatments applied. Thus, oOn the basis~~
390 ~~of the medium-term (one year) results, Preventol RI80® proved to be the most suitable~~
391 ~~effective treatment, followed by benzalkonium chloride and the ethanol-water (1:1)~~
392 ~~mixture, which yielded similar results-, and then by Biotin R® and Biotin T®. New~~
393 ~~Des 50® had a deleterious effect, with clearly visible colour changes on the granite~~
394 ~~substrate, and was thethe least effective response-againsteffective in removing -the~~
395 ~~subaerial biofilm formed by *Apatococcus lobatus* (Chodat) J.B.Petersen (Chlorophyta).~~
396 ~~Based oOn the basis ofn the results achieved after one year of study and concerns about~~
397 ~~the sustainability-potential toxicity of the products tested, the recommendation however~~
398 ~~if thewe recommend that if the-elimination-of biofilm has to be removed infrom the~~
399 ~~cloister wall is mandatoryobligatory (for whatever reasons beyond the investigation), is~~
400 ~~the treatmentthis should be done with water and brushing, as was applied in thise~~
401 ~~present workstudy.~~

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570 Figure 1. Monastery of San Martiño Pinarío. (A) Main entrance. (B) Central patio of the
571 processional cloister. (C) Map of the cloister: the location of the trial cleaning area is
572 indicated by a green rectangle. (D) West-facing wall, where the trial cleaning areas were
573 located.

574

575 Figure 2. Trial cleaning areas on the west-facing wall of the Processional cloister. The
576 white outer frame delimits an area of ca. 400 cm² used for colour measurements and the
577 white inner frame delimits the area of ca. 100 cm² used for chlorophyll fluorescence
578 measurements. Each of these areas was divided into three smaller, replicate areas: A, B,
579 C in the large area and a, b, c in the small central area.

580

581 Figure 3. Changes in the green-red colour component (Δa^*) and blue-yellow colour
582 component (Δb^*) in the trial areas throughout the study period. Bars represent the
583 means \pm standard errors of three replicate areas. Different letters indicate significant
584 differences between the results achieved in the nine trial areas (including a reference,
585 uncolonized area) for each of the times the same time (i.e., at 1, 3, 6 or 12 months),
586 determined by post hoc comparisons (Duncan's test, * $p < 0.05$).

587

588

589 Figure 4. Values of F_v/F_m , the maximum quantum efficiency of photosystem II (PSII)
590 photochemistry, at 665 nm. Bars represent the means \pm standard errors of three replicate
591 areas. Different letters indicate significant differences between the results achieved in
592 the nine trial areas (including a reference, uncolonized area) for the same time (i.e., at 1,
593 3, 6 or 12 months) each of the times, determined by post hoc comparisons (Duncan's
594 test, * $p < 0.05$).

595

596 Figure 5. Chlorophyll-a content and phaeophytinization quotient, which reflects the
597 ratio of chlorophyll-a to phaeopigments (chlorophyll-a degradation products), in the
598 trial areas at the end of study period (12 months). Bars represent the means \pm standard
599 deviations of three replicates. Different letters indicate significant differences between
600 the results achieved in the nine trial areas (including a reference, uncolonized area)
601 determined by post hoc comparisons (Duncan's test, * $p < 0.05$). The concentration of
602 chlorophyll-a in the colonized area was $2.31 \pm 0.08 \cdot 10^{-2} \mu\text{g cm}^{-2}$ (not shown in the
603 graph).

604

605 Figure S1. Cumulative average values for parameters L^* , a^* , b^* in relation to the
606 number of measurements, which indicate the minimum number of colour measurements
607 required (Prieto et al., 2010a). Chart of results and example of the graphs used to
608 determine them, with marked segments indicating the minimum number of
609 measurements.

610