Species- and site-specific efficacy of commercial biocides and application solvents against lichens

SPECIES- AND SITE-SPECIFIC EFFICACY OF COMMERCIAL BIOCIDES AND APPLICATION SOLVENTS AGAINST LICHENS

Favero-Longo Sergio E. *[a], Benesperi Renato [b], Bertuzzi Stefano [c], Bianchi Elisabetta [b], Buffa Giorgio [a], Giordani Paolo [d], Loppi Stefano [e], Malaspina Paola [d], Matteucci Enrica [a], Paoli Luca [e], Ravera Sonia [f], Roccardi Ada [g], Segimiro Alessandro [a], Vannini Andrea [e]

[a] Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Viale Mattioli 25, 10125, Torino, Italia
(sergio.favero@unito.it; giorgio.buffa@unito.it; enrica.matteucci@unito.it; alessandro.segimiro@unito.it)

[b] Dipartimento di Biologia, Università di Firenze, Via La Pira 4, 50121, Firenze, Italia
(renato.benesperi@unifi.it; bnclbt@unife.it)

[c] Dipartimento di Scienze della Vita, Università di Trieste, Via Giorgieri 10, 34127, Trieste, Italia
(sbertuzzi@units.it)

[d] Dipartimento di Farmacia, Università di Genova, Viale Cembrano 4, 16148, Genova, Italia
(giordani@difar.unige.it; pmalaspina81@gmail.com)

[e] Dipartimento di Scienze della Vita, Università di Siena, Via Mattioli 4, 53100, Siena, Italia
(stefano.loppi@unisi.it; luca.paoli@unisi.it; andrea.vannini@unisi.it)

[f] Dipartimento di Bioscienze e Territorio, Università del Molise, C. da Fonte Lappone, 86090, Pesche (IS), Italia
(sonia.ravera@unimol.it)

[g] Istituto Superiore per la Conservazione ed il Restauro, Via di San Michele 23, 00153, Roma, Italia
(ada.roccardi@beniculturali.it)

*Corresponding author:
Sergio E. Favero-Longo, PhD.
Università degli Studi di Torino
Dipartimento di Scienze della Vita e Biologia dei Sistemi
Viale Mattioli 25, 10125 Torino, Italy
Tel. +390116705972
Fax +390116705962
sergio.favero@unito.it

Abstract

Control of lichens on stone cultural heritage is mostly achieved by a combination of mechanical removal with biocide applications. However, there is a lack of scientific evidence on the efficacy of different biocides on different species, and on the consistency of biocide effects on heritage sites in different environmental conditions. This results in some uncertainty when conservation interventions to control lichens are routinely defined on the basis of restoration tradition or empirical evaluation, without experimental measures of how lichens respond. In this work, we quantitatively evaluated (a) the efficacy of five commercially-available biocides, applied using a brush or with a cellulose poultice, against two species (*Protoparmeliopsis muralis*, *Verrucaria nigrescens*), and (b) whether the effects on the two species were consistent, per treatment, across three Italian heritage sites. Lichen vitality was quantified through analyses of chlorophyll *a* fluorescence (Chl_aF) and ergosterol content. The results indicated that all the tested biocides, and their organic solvents, affected the vitality of both the species. However, most of treatments displayed different efficacy on each species, across the different sites and between brush and poultice applications. Accordingly, when a conservation intervention to control lichen growth is planned, biocide treatments need both species- and site-specific calibrations and lichen vitality should be properly ascertained *in situ* by monitoring Chl_aF parameters (F_V/F_M and F_0) twenty days after trial biocide applications.

Keywords

biocide; chlorophyll *a* fluorescence; ergosterol; lichen; organic solvents

1. Introduction

The effects of lichens on stone monuments are nowadays considered a matter of debate, as researchers are increasingly contributing, and counterposing, evidence for lichen-related biodeterioration and bioprotection processes (Salvadori and Casanova-Municchia 2016). The need to remove lichens in all cases may be reasonably questioned, as for example in cases where lichen colonization accounts for a negligible deterioration effect, shows some bioprotective attributes, contributes to the aesthetic of the monument and/or represents biodiversity value (Pinna 2014). Nevertheless, in cultural heritage management a direct relationship between lichens and weathering is still usually envisaged, and lichen removal is generally planned as component of restoration interventions (Caneva et al. 2008).

In any cleaning interventions, devitalization of lichens is necessary to avoid them being undesirably scattered, rather than controlled, by the cleaning actions (Caneva et al. 2008). So far, the application of biocides has been the most followed approach to kill lichens, although chemical treatments give rise to concerns about their impact on the environment (e.g. Gromaire et al. 2015) and have already showed technical limitations (Speranza et al. 2013 with refs therein). Biocide application has indeed yielded mixed results, including poor treatment response, changes in community dynamics, persistence of dead thalli, and damage to substrate surfaces (Seaward 2015). Accordingly, several innovative and promising approaches have been proposed in the last years to substitute for, or reduce, biocide application, including heat shock treatments (Tretiach et al. 2012), infrared and ultraviolet laser irradiation (Speranza et al. 2013; Sanz et al. 2015; Pozo et al. 2016), and others, which still need to be better calibrated on lichens, such as anatase photocatalysis (Fonseca et al. 2010) or enzymatic treatments (Scarpa et al. 2016). Nevertheless, the adoption of these new techniques is generally limited by experimental time, extent of surfaces to be treated, and, in some cases, economic constraints, while the use of biocides persists as a routinely adopted approach, with protocols often based on traditions and empirical evaluations more than on experimental analyses of their efficacy in each case-study (Caneva et al. 2008).

Research on biocidal effects on lichens has been conducted since the 1970s and 1980s, with treatment success being mostly empirically defined *in situ* (Caneva et al. 1996, and references therein), while standardization of experimental techniques to assess lichen devitalization after biocide application (i.e. fluorescence microscopy) was established at the beginning of 1990s (Normal 1994). Conservators have claimed some difficulties in directly testing a range of biocide and cleaning agents (Schnabel 1991), and have noted the need for comprehensive reviews on commonly used biocidal materials (e.g. Caneva et al. 1996). However, as a response, lists of products rather than investigations into their efficacy have been produced, and some products have become outdated over the years, following the recognition of their toxicity-related environmental and health hazards (Nugari and Salvadori 2003; European-Commission-Regulation 2007; SCENIHR 2009). More recent research has considered the biocidal effect(s) of restricted sets of products (e.g. Tretiach et al. 2007; de los Ríos et al. 2012), in comparison with physical treatments (e.g. Fonseca et al. 2010; Tretiach et al. 2012) or in combination with other restoration products (e.g. Pinna et al. 2012). Different approaches to assess the effects of the treatments have been considered, including microscopical observation of chlorophyll epifluorescence in photobionts (Nugari et al. 1993), SEM evaluation of the integrity of anatomical structures of both lichen

partners (Speranza et al. 2012), fluorimetric analyses of biophotonic activity (Bajpai et al. 1992) and chlorophyll *a* fluorescence of photobionts (Chl_aF) (Tretiach et al. 2008, 2010), electrical conductivity of thalli (Cuzman et al. 2013) and molecular assessments (e.g. DGGE; Cámara et al. 2011). The diversity of methods used to assess lichen devitalization in these studies makes it hard to compare results. Moreover, although a species-specific lichen sensitivity to biocides has been suggested (Alstrup 1992; Nimis and Salvadori 1997), only few researchers have included a focus on this feature (Tretiach et al. 2007, 2010, 2012). More remarkably, researchers have neglected to evaluate the *in situ* reproducibility of devitalization results across different heritage sites, nor have they clarified if different biocidal approaches, in terms of active principle, preparation solvent and/or application method, may be more or less suitable against certain species, on certain stone substrates or under certain macro- and micro-climatic conditions. However, similar information, in parallel with research on alternative approaches for lichen control, would be of value to optimize routinely-adopted biocidal application, and, consequently, reduce related environmental contamination (Scheerer et al. 2009).

In this research, we compared the effects of five commercial biocides, nowadays widely used in Europe (BiotinR, BiotinT, DesNovo, Lichenicida 464, Preventol RI80), and their application solvents (water, acetone, White Spirit) on the vitality of two epilithic lichens [*Protoparmeliopsis muralis* (Schreb.) M. Choisy and *Verrucaria nigrescens* Pers.] commonly found on stone cultural heritage in Europe and beyond (Nimis et al. 1992). The effects of the herbicide glyphosate (Glifene SL) and of the lichen secondary metabolite usnic acid, having biocidal potential against other deteriogenic lithobionts (Gazzano et al. 2013), were also assayed. All the products were applied *in situ*, with single brush and poultice applications at concentrations following the producers' recommended ranges, on lichen thalli growing on sedimentary rocks in three Italian heritage sites located in different (phyto-)climatic areas (as defined in Nimis and Martellos 2008). The research did not aim to rank the performance of the different products, as each product was not tested in all possible concentrations, application methods and treatment cycles. The aims of the study were to quantify, for a series of biocide treatments, (a) if each approach (i.e. biocide × application method) showed a similar efficacy against different lichen species, and (b) if efficacy results were consistent, per species per treatment, between different sites. To accomplish these aims, we examined in each study site the vitality of lichen thalli before and after the treatments in terms of chlorophyll *a* fluorescence (Chl_aF) of the photobiont, recognized as an ideal tool for checking the vitality of photosynthetic organisms, including lichens (Tretiach et al. 2012; Malaspina et al. 2014). Additional analyses were also, in turn, performed to clarify the lichen response to biocide treatments, including microscopic assessment of chlorophyll epifluorescence in photobionts and the assessment of mycobiont vitality in terms of ergosterol content.

2. Materials and methods

2.1 Sites and lichen species

Biocide applications on lichens were performed, *in situ*, at three heritage sites distributed in different (phyto-)climatic areas of Italy: (A) the Roman Archaeological site of Industria [Monteu da Po, Torino; UTM ED50, N 5001078, E 422890; 170 m], in the dry sub-Mediterranean area; (B) the Roman Archaeological site of Luni [Ortonovo, La Spezia; UTM ED50, N 4879338, E 581882; 3

m], in the humid Mediterranean area; (C) the Boboli Gardens [Firenze; UTM ED50, N 4847851, E 680788; 49 m], in the humid sub-Mediterranean area (Fig. S1). Treatments were performed on mature thalli of the epilithic crustose placodiomorph *Protoparmeliopsis muralis* (Schreb.) M. Choisy and the epilithic crustose areolate *Verrucaria nigrescens* Pers. (Fig. S1), which were identified following Smith (2009). These two subcosmopolitan species are extremely common both in urban and natural habitats (Nimis and Martellos 2008), and on stone cultural heritage (Nimis et al. 1992). In particular, 60 thalli per species for each site were selected and treated: (A) on local sandstone masonry blocks at Industria, (B) on sandstone (Macigno sandstone from Lunigiana) blocks, and the adjacent mortar, at the amphitheatre of Luni, and (C) on the sandstone (Pietra Serena) pavement slabs of the monumental Fontana dell'Isola in the Boboli Gardens, at approx. 50 cm from the fountain water.

2.2 *Biocide application*

Biocides were applied by a professional restorer (site A) or under his supervision (sites B and C). Each biocide was prepared following the manufacturer's instructions (Table 1, including biocide abbreviations) and applied, (i) using a paint-brush and (ii) with a cellulose poultice, using similar quantities of biocide and after having moistened the thalli with sprayed water (Fig. S1). The applied cellulose poultice was kept covered with a cotton fabric for four hours and then gently removed with a small spatula. After the four hours, all brush- and poultice-treated thalli were gently washed with water. The solvents recommended for biocide dilution (water, acetone, White Spirit) were also separately tested. Bottled water with low salt contents was used for all experiments, to avoid any salt-induced reduction in biocide efficiency (Caneva et al. 1996). Three thallus replicates per species per biocide per application method were examined.

Daily meteorological data (air temperature, relative humidity, rainfall) for the week preceding and the three weeks following the biocide application at the three sites (A, October 2015; B, April 2016; C, May 2016) were obtained from nearby monitoring stations: A, Verolengo station (ARPA Piemonte 2016), B, Luni station (ARPA Liguria 2016), C, Firenze-Lamma station (ARPA Toscana 2016).

2.3 Lichen vitality measurements

Chlorophyll *a* fluorescence measurements (Chl_aF) were carried out *in situ* one day before (T0), and one (T1) and 20 (T20) days after the biocide treatments, using a Handy-PEA fluorimeter (Plant Efficiency Analyser, Hansatech instruments Ltd., Norfolk, England). Analyses were performed early in the morning on dark-adapted moistened thalli, previously sprayed with bottled water and covered overnight with a black cotton fabric. Fifteen minutes before each measurement, thalli were again sprayed and covered. Five measurements were taken on each thallus, positioning the sensor head, equipped with three light emitting diodes (LED), at 90° over its surface and avoiding, in the case of *P. muralis*, areas covered by apothecia. Chl *a* fluorescence was induced by a red light (peak at 650 nm) and data recorded after a saturating light pulse of 1 s (Malaspina et al. 2014). Chl_aF increases from F_0, when all the reaction centres (RCs) of Photosystem II (PSII) are open, to F_M, when all the RCs of PSII are closed. The maximum quantum efficiency of PSII, that is F_V/F_M (where $F_V=F_M-F_0$), a temperature-independent parameter of Chl_aF emission, was used to check the vitality of the thalli (Tretiach et al. 2012).

184 The analysis of F_V/F_M was combined with a wider evaluation of the OJIP transient, the polyphasic
185 transient exhibited by Chl_aF when plotted on a logarithmic time scale, and of F_0 values. The shape
186 of the OJIP curves is informative on the structure and function of the photosynthetic apparatus
187 (mostly related to PSII) (Malaspina et al. 2014), while F_0 is related to the chlorophyll contents of
188 the light harvesting complex (Baruffo and Tretiach 2007). In site B, additional parameters of the
189 OJIP analysis, including the number of reaction centres (RC), the energy flux trapped by the
190 reaction centres (TR) and the energy flux dissipated as heat (DI), were also considered as indirectly
191 informative on the structure and function of the photosynthetic apparatus upon exposure to stress
192 factors (Malaspina et al. 2014, 2015). All these data were referred to excited cross sections (CS) of
193 the examined lichen, determined by the area of the thallus subjected to the light impulse emitted by
194 PEA (Malaspina et al. 2014).

195 At site A, Chl_aF measurements were also performed 180 days after the biocide treatments (T180)
196 and combined with epifluorescence observations of hand-made cross-sectioned thalli, carried out
197 under a Nikon Eclipse 300 microscope. Quality and quantity of the fluorescence emitted by
198 photobiont cells, spatially informative on the vitality of the photobiont layer (e.g. Pinna et al. 2012),
199 were evaluated, and the data interpreted using an ordinal scale on the relative abundance of viable
200 (red coloured) and devitalized (appearing white) cells.

201 At site C, the analysis of Chl_aF in the photobiont of *P. muralis* was combined with analysis of
202 ergosterol content in the mycobiont. Ergosterol is indeed the main sterol of the mycobiont plasma
203 membranes and its content is correlated with basal respiration rates and cell membrane integrity
204 (Sundberg et al. 1999). Analyses were performed as previously described by Vannini et al. (2016).
205 In brief, thallus fragments (100 mg) were homogenized for 10 min in 99% ethanol, and the extracts
206 were shaken in the dark at 25 °C for 30 min, then vortexed and centrifuged at 10,000 g for 20 min.
207 The resulting supernatant was immediately analyzed by HPLC (Hitachi 665A-12 with LC
208 Controller L-5000) using a Phenomenex C18 column (150 x 4.6 mm^2; particle size 5 μm) at a flow
209 rate 0.8 mL/min and isocratic elution with methanol as mobile phase. Total analysis time was 15
210 min. Absorbance at 280 nm was measured with a UV detector (Jasco 875/UV). A standard curve
211 was prepared ranging 1–200 mg ergosterol from Sigma-Aldrich (USA) dissolved in 1 mL of
212 ethanol. Two replicates were measured for each sample.

213 *2.4 Statistics*

214 Generalized Linear Models (GLMs) were applied for each lichen species to describe the effects of
215 the treatments on photobiont vitality (F_V/F_M). For each model we set biocide (water *vs*. different
216 biocides), time (T0 *vs*. T1, T20), application method (brush *vs*. poultice), and site (site C *vs*. sites A,
217 B) as fixed factors. Second level interactions between biocide, time, application method and site
218 were also considered. GLM analyses were carried out with R (R Development Core Team 2010),
219 using glmer function of lme4 package (Bates et al. 2014). A factorial ANOVA analysis was
220 performed to detect significant differences in F_V/F_M according to the different predictors (biocide,
221 time, application method, site). Calculations were performed using the software package Statistica
222 Version 8.0 (StatSoft, Tulsa, OK).

223 For each study case, significant differences in F_V/F_M related to time and respect to a threshold
224 (arbitrarily fixed at $F_V/F_M = 0.15$, as discussed in section 4.2) were analyzed by means of ANOVA

with post-hoc Tukey's and t-test, respectively, using SYSTAT 10.2 ($P<0.05$ as significant). For each study case, decreases of F_0 at T1 and T20, with respect to T0, significantly higher than 80%, were assessed by means of ANOVA with post-hoc t-test ($P<0.05$ as significant). Data on the other parameters of the OJIP analysis and ergosterol contents were analyzed by means of ANOVA with Tukey's post-hoc test.

3. Results

GLM tests (Table 2) indicated, for each species, rather uniform F_V/F_M values before the biocide treatments. Slightly higher values characterized *P. muralis* at site A, and lower values occasionally characterized individuals of *V. nigrescens* (in particular, the individuals used to assay BR and WS at site C): however, these values were still within the expected range of variability in viable thalli of this species (Speranza et al. 2012). When treated with water (control), both species did not show variations of F_V/F_M according to time (T0 *vs*. T1 and T20). Significant differences upon application of all biocides and organic solvents were evident at T1 and T20 (DN, PV, BT, BR, LI, WS, AC × T1, T20). UA did not affect F_V/F_M, while GL only affected *P. muralis* at T20. Biocide application with cellulose poultice was more effective than brushing in reducing F_V/F_M.

For each species, the range of variability observed in the predictor estimates calculated for the biocide and solvent treatments (at T20: from -0.51 to -0.24 in *P. muralis*, and from -0.38 to -0.15 in *V. nigrescens*) indicated some further differences in their effectiveness. Figures 1 and 2 show F_V/F_M values for the two species at T1 and T20, and percentage decrease in F_0 values with respect to T0, respectively. Only in a few cases did the significant decrease of F_V/F_M with respect to controls correspond to the complete zeroing of the parameter (i.e. 0.00). However, for both species, all biocides (DN, PV, BT, BR, LI) and the two organic solvents (WS, AC) were able to induce, at least in some study cases (application method × site), a decrease in F_V/F_M below the threshold fixed at 0.15, putatively indicative of the loss of vitality of the photobionts (as discussed in section 4.2).

In *P. muralis*, the decrease of F_V/F_M at T1, including some values below 0.15, was associated with a moderate decrease in F_0; while at T20, most F_V/F_M values below 0.15 were associated with a strong decrease in F_0 (>80% in 82% of cases). In other cases, similarly strong F_0 decreases at T20 were associated with slight signals of F_V/F_M recovery from T1 to T20 (at site C), and, occasionally, with minor lowering of F_V/F_M.

In *V. nigrescens*, the relationship between F_V/F_M values below 0.15 and the strong decrease in F_0 (>80%) was restricted to a more limited set of cases (mostly the poultice treatments at site A). In some others, as described for *P. muralis*, strong decreases in F_0 were related to a moderate lowering of F_V/F_M or, at site C, to slight signals of recovery. Differently, at site B, values of F_V/F_M below 0.15 were associated with relatively poor decreases in F_0 (30-60%).

OJIP transients (Fig. 3) clarified that in all cases the strong decreases of F_V/F_M (<0.15) and/or F_0 (>80%) reflected a substantial loss of vitality of the photobiont. At site C, those slight increases of F_V/F_M from T1 to T20, simulating a partial recovery of both the species (e.g. for BR and LI), depended on ground noise of Chl_aF around zero (Fig. 3C, D). The low percentage decrease of F_0 in *V. nigrescens* at site B was associated with lower initial (T0) values than in other sites (Fig. 3B).

In *P. muralis* thalli which displayed strong decreases of F_V/F_M (<0.15) and/or F_0 (>80%) parameters, the number of reaction centres (RC_0/CS) and the trapped excitons (TR_0/CS) dropped already at T1 and did not recover at T20 (Table S3). The heat dispersion (DI_0/CS) increased at T1, and then remarkably dropped at T20. Other thalli displayed a gradual and less pronounced decrease of RC_0/CS and TR_0/CS; they also displayed a relative DI_0/CS increase, which, however, was not followed by dropping at T20.

Thalli of *V. nigrescens* strongly affected in F_V/F_M and F_0 also showed a fast drop of RC_0/CS and TR_0/CS (with the exception of DN samples, for which the parameters dropped at T20) and the increase of DI_0/CS at T1, which however mostly recovered initial low values at T20 (Table S4). In thalli displaying no or minor variations in F_V/F_M and F_0, parameters RC_0/CS, TR_0/CS and DI_0/CS were not significantly affected.

Epifluorescence observations run on thalli from site A at T180 also displayed some differences between the two species in the photobiont response to biocides (Table S5). Thalli of *P. muralis* which showed a decrease of F_V/F_M values below 0.15 did not exhibit any living photobiont cell. By contrast, in *V. nigrescens*, even the thalli with F_V/F_M below 0.15, and a decrease of F_0 by 98-99%, still showed some residual viable cells in the lower part of the photobiont layer. In parallel, for both the species, analyses carried out at T180 showed that results evaluated at T20 were mostly reliable indicators of the long-term response of the thalli to the biocide treatments: only in one out of the 40 treatments (AC applied to *P. muralis* by brush), F_V/F_M values at T20 and T180 displayed a different position with respect to the threshold fixed at 0.15.

To evaluate the consistency of treatment effects across the different sites, the strong decreases of F_V/F_M (<0.15) and/or F_0 (>80%) were considered indicative of the loss of photobiont vitality (while residual vitality microscopically observed in *V. nigrescens* was disregarded). At least for some "biocide × application method" cases, results obtained at the three sites showed differences (Table 3), possibly due to contrasting environmental conditions. A potential influence of meteorological conditions preceding, during and following the biocide application (Table S6 in Supplementary Materials) was considered. Biocide application in site C was performed during a rainy day, and surfaces were provisionally protected with a plastic canopy and a tarpaulin. Precipitation rates in the weeks preceding and following the biocide application were rather comparable for the three sites, while air temperature (T) and humidity (RH) showed relatively slight, but significant (ANOVA, $P<0.05$) differences, with T in site $A \leq B \leq C$ and RH in $A \geq B \geq C$.

Finally, ergosterol content (Fig. 4), although limited to *P. muralis* at site C, reflected the damage endured by the lichen mycobiont, and suggested further patterns of variability in the potential effectiveness of the different biocidal approaches (biocide × application method). Significant decreases with respect to the water controls were detected for biocides PV, BR and LI and the organic solvent WS, their effect also depending on the application method. Moreover, a decrease in ergosterol content was observed for thalli treated with UA when applied with cellulose poultice.

4. Discussion

Review of the literature suggested that the same active principle may have different levels of biocidal performance in relation to different intrinsic (concentrations, solvents, solution pH, duration of the application) and extrinsic (nature and conservation of the substrate, colonization extent, microclimate) parameters (Caneva et al. 1996, 2008). In the case of lichens, early empirical evaluations (see Caneva et al. 1996) have been poorly supported with quantitative comparative evaluations of intra- and interspecific variability through different case studies.

In this work, we showed the sensitivity of *P. muralis* and *V. nigrescens* to treatments with five widely used biocides, replicated on sandstone surfaces at three heritage sites. Analyses of Chl_aF, quantified as vitality of the photobiont (Tretiach et al. 2010, 2012), demonstrated a significant efficacy of all products, including biocides and their organic solvents, against both species. However, the investigation also displayed for each treatment different levels of efficacy against the two species and/or across the different sites, exposed to slightly different meteorological conditions in days following the biocide applications, and specific patterns of residual vitality.

4.1 Performance and timing of biocidal applications

The tested products are known to exert different biocidal mechanisms: quaternary ammonium compounds, including benzalkonium chloride (DN, PV), interfere with biological membranes by changing their structure and permeability; isothiazolinones (BT, BR, LI) oxidate thiol-containing cytoplasmic and membrane-bound compounds, yielding metabolic inhibition (Denyer and Stewart 1998). Biocides rapidly (T1) reduced the functionality of the photosynthetic process in both tested species, while limited effects (T20) were observed for GL on *P. muralis*, and almost no effect for UA. In laboratory experiments, the same commercial glyphosate solution, at the same concentration (3 times higher than the highest suggested dose), determined a zeroing of F_V/F_M in *Xanthoria parietina* (Vannini et al. 2016), suggesting that different lichen species can differently tolerate this herbicide, which targets a key enzyme (5-enolpyruvyl-shikimate-3-phosphatesynthase, EPSPS) involved in the production of the aromatic aminoacids (phenylalanine, tyrosine and tryptophan), or that, alternatively, laboratory conditions may not properly mimic glyphosate applications on stone materials *in situ*. UA, which was effective to control the growth of other biodeteriogens, such as cyanobateria and microcolonial fungi (Gazzano et al. 2013), did not affect the *Trebouxia* photobionts of *P. muralis*, a lichen which produces UA (Smith et al. 2009). No effects of UA were also observed on the photobiont partners of *V. nigrescens*, (e.g. *Diploshphaera* sp.; Thus et al. 2011), which does not secrete lichen secondary metabolites. On the other hand, effects of UA on the mycobiont of *P. muralis* suggest a potential role of the secondary metabolite in autoallelopathic processes, poorly explored for lichens and certainly far from being exploitable to control lichens on stone cultural heritage.

Remarkably, we observed a significant effect of pure organic solvents (AC, WS) in the inhibition of photosynthetic processes of both the species. A different AC tolerance of different lichen species was already known (Solhaug and Gauslaa 2001): in laboratory experiments, the time of immersion in AC required to zero F_V/F_M in different lichen species ranged from few hours, compatible to our field treatments, to hundreds of hours (Solhaug and Gauslaa 2001). A higher level of tolerance was attributed to *Trebouxia*-bearing species displaying a high drought resistance, as AC may exert a negative effect by extracting residual water from the dried thalli (Solhaug and Gauslaa 2001). However, AC does not easily pass or destroy the membranes when cells are desiccated (Solhaug

346 and Gauslaa 2012), while AC was here applied to wet thalli. Accordingly, *P. muralis* and *V.*
347 *nigrescens* appeared similarly affected by AC, and comparable effects were also observed upon WS
348 treatments.

349 Biocides were applied after wetting thalli and during humid seasons (Autumn in site A, Spring in
350 sites B and C), when significant rain events, as expected in (sub-)Mediterranean areas, similarly
351 occurred in all the experimental sites, and high values of RH% were generally recorded (see Table
352 S6). Although seasonal variations are known for F_V/F_M in lichens (Baruffo and Tretiach 2007),
353 measurements at T0 generally indicated an overall healthy state of thalli before the biocide
354 application. Effective biocidal effects were already recognizable at T1, in terms of F_V/F_M, RC_0/CS
355 and TR_0/CS decreases, and only for some brush applications of biocides dissolved in water
356 significant decreases were observed later, from T1 to T20. Increased DI_0/CS values at T1 also
357 indicated a fast biocidal activity, as photobionts rapidly attempted to increase controlled de-
358 excitation processes, as thermal energy dissipation, to avoid oxidative damage related to an affected
359 electron flow in the photosynthetic apparatus, as reported under other stress condition (Malaspina et
360 al. 2015).

361 Since early empirical observations, more noticeable effects of biocides have been recognized when
362 they were applied to wet thalli or when rain events followed their application, while effects have
363 also been observed after some months when biocides were applied in arid seasons (Nimis and
364 Salvadori 1997). When poikilohydric organisms, like lichens, are dehydrated, their structures and
365 macromolecules are protected by glass-like matrices and can tolerate extreme conditions (Tretiach
366 et al. 2012; Fernandez-Marin et al. 2013), including biocide application (Alstrup 1992).
367 Accordingly, the slow-rate effect observed in this study for water-dissolved biocides applied by
368 brush likely depends on the fact that thalli were more rapidly air-dried than those treated with
369 poultices and remained active for a shorter period after the treatment. Moreover, water retention by
370 the porous sandstone lithologies may have contributed to absorb and dilute the brush-applied,
371 water-dissolved biocides (Caneva et al, 1996), limiting a rapid effect.

372 The application method did not only influence the timing, but, in general, also the biocide
373 performance, with the highest efficacy mostly detected for poultice applications being reasonably
374 explained by an increased contact time between biocide, hydrated thalli, and sandstone substrates
375 (Nugari and Salvadori 2008; Pinna et al. 2012). This may increase rapid effects (observed at T1),
376 but also successive ones, by enhancing biocide retention within substrate, and possibly limiting its
377 washing out under rainy conditions (Young et al. 1995; Caneva et al. 1996; Cameron et al. 1997). In
378 our work, this was evident for the applications of biocides dissolved in water, while minor
379 divergence between the effects of brush and poultice applications was detected for those prepared
380 with organic solvents. Reduced efficacy and recovery were observed when solvents alone were
381 applied by brushing, likely because of the rapid evaporation of the products.

382

383 *4.2 Chl_aF measures and specific residual vitality*

384 The effect of different biocides was associated with different persistence of thallus remnants on the
385 stone surfaces or penetrating structures within the substrate after the mechanical cleaning (de los
386 Ríos et al. 2012). However, scarce information exists to evaluate whether lichen recolonization

depends on the arrival of new propagules dispersed by external populations (Favero-Longo et al. 2014; Morando et al. in press), or whether mechanical cleaning enhances the spreading of living remnants of partially killed thalli, as documented during attempts to remove thalli without previous devitalization (Seaward 2004, 2015). Cases of recolonization of restored surfaces by lichen communities different from those occurring before the treatments suggest external inputs (Nascimbene et al. 2009). Nevertheless, in other cases, the persistence of lichen remnants after cleaning was correlated with short-term re-increases of lichen cover (e.g. 16 months after the application of isothiazolones combined with benzalkonium chloride or other active principles, in Cámara et al. 2011).

In our investigation, the analysis of F_V/F_M was combined with OJIP transients and F_0 variations with the aim of offering a straightforward information on the death of lichen thalli. A threshold of F_V/F_M values reflecting dead photobionts has not been explicitly stated in literature, although dips below 0.100-0.200 often reflect dead material (e.g. Solhaug and Gauslaa 2001; Speranza et al. 2012; Tretiach et al. 2012). Accordingly, we accounted for a threshold at F_V/F_M=0.150, strictly associated with a dropping of the number of reaction centres (RC_0/CS) and trapped energy fluxes (TR_0/CS).

An overview on the OJIP shapes at T20, with a focus on the starting base fluorescence of the curve (F_0), combined with the quantification of the F_V/F_M parameter, helped to check for the residual vitality of the biocide treated thalli. In particular, a strong % decrease of F_0 values (threshold tentatively fixed at -80%), associated with the flattening of curve and the dropping of RC_0/CS and TR_0/CS parameters, appears an additional marker of death. Slight F_V/F_M recoveries, as those observed for both the species in site C, appear irrelevant (as related to measuring noises) when calculated on flat transients with zeroed F_0 values. F_0 variations are primarily related to chlorophyll contents of the light harvesting complex (Baruffo and Tretiach 2007), which are fatally or, at least, severely affected when flat and zeroed curves are calculated.

In the light of these discussed Chl_aF parameters, we can finally consider (at least for the poultice applications) that all biocides, and the organic solvents, were effective in killing both the species at least in one of the sites (see Table 4). However, *P. muralis* and *V. nigrescens* were not strongly/fatally affected in 14% and 24%, respectively, of poultice applications of biocides and organic solvents, and displayed different sensitivity to the same treatment (i.e. significant decrease of F_V/F_M and/or F_0 below the thresholds, or not) in 29% of examined cases (biocide × site; see Table 3). Moreover, epifluorescence observations carried out at T180 suggested some higher resistance for *V. nigrescens*, as some residual healthy photobiont cells were observed in the lower part of the photobiont layer, even in thalli for which both F_V/F_M and F_0 decreased below the fixed thresholds. Accordingly, *V. nigrescens* already showed more resistance to cleaning treatments than another compared species [*Lecidella stigmatea* (Ach.) Hertel & Leuckert] after the application of Biotin R (de los Ríos et al. 2012). Thalli of *P. muralis* are thicker than those of *V. nigrescens*, and also display a thicker cortex. However, the very tightly packed, short-celled hyphae of the paraplectenchymatous cortex of *V. nigrescens*, with melanin in the uppermost layers, may determine a lower permeability with respect to the different cortex type of *P. muralis* (cone-cortex; see Büdel and Scheidegger 2008), covered by the hydrophobic, but organic solvent-soluble usnic acid (Smith 2009).

Differences across the three sites in the sandstone substrates, possibly related to different physical and chemical properties as suggested by the extreme variability between and within each sandstone type (e.g. Franzini et al. 2007; Fratini et al. 2015), may primary account for the efficacy variability observed for certain treatments. Different porosity, capillary water absorption capacity and mineral composition of different sandstones can indeed affect the quantity of biocide absorbed by the substrate and its effective life span in a bioactive form (Young et al. 1995; Cameron et al. 1997). The substrate chemical composition also influences the performance of some biocides, including the quaternary ammonium salts (Caneva et al. 1996). In parallel, different meteorological conditions of each site in the days following the biocide applications could also account for some variability (Nimis and Salvadori 1997). However, cases of poor efficacy were randomly distributed rather than clearly related to any of the heritage sites. Relevance of the variability in T, RH, and sandstone properties between sites on the biocidal mechanism of each product may be hypothesized (Caneva et al. 1996), but should be tested on a wider set of heritage sites, and is beyond the aim of this work. Influences of other environmental conditions, including microclimate differences (Caneva et al. 2008), but also biocide-specific population resistances, should be also worth of investigation.

Accordingly, waiting for further research to address reliable models to predict the suitability of a biocide against certain species in certain environmental conditions, species- and site-specific calibration of biocidal strategies is necessary. Such calibration would likely exclude the usage of products, concentrations or application methods which may not show the desirable killing efficacy, and would strongly reduce the dispersal of fragments with residual vitality during the mechanical procedures which follow the chemical treatment. If biocides are applied to wet thalli, their efficacy can be confirmed within few weeks (T20) by Chl_aF measurements. Pilot biocide assays appear thus compatible with the time pressure which often characterizes restoration interventions. On the other hand, our results highlighted how the different biocides may also differently affect the mycobiont (as shown by ergosterol content), suggesting the opportunity of conducing controlled experiments on the potential recovery trends on the medium and long term of both the symbiotic partners and their joined influence on the recolonization potential of the different lichen species.

5. Conclusions

On the basis of quantitative Chl_aF measurements, our work showed that (a) different biocidal approaches (product × application method) may affect the vitality of lichens at a heritage site, however with different efficacy against each species. Moreover, our findings indicate that (b) the efficacy of a biocidal treatment against a lichen species cannot be assumed to be consistent across different heritage sites. Such complexity suggests that if a biocide approach is planned to manage lichens on a stone surface, *in situ* pilot assays to calibrate biocidal treatments on the particular study case (species × site) should be run.

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625

626 **Tables**

627 Table 1 - Biocides and solvents applied in the experiments

Commercial product		Producer	Active principle	Solvent	Recommended concentration	Used concentration
Water	W	-	-			-
Glifene SL	GL	Chmiberg-Diachem (Caravaggio, Italy)	Glyphosate [i.e. N-(phosphonomethyl)glycine; 30-40%] in water	Water	0.003 - 0.011%	0.04%
DesNovo	DN	Bresciani S.r.l. (Milano, Italy)	Benzalkonium chloride (i.e. alkyl dimethyl benzyl ammonium chloride; 10%) in water	Water	0.5 - 10%	2.00%
Preventol® RI80	PV	Lanxess (Köln, Germany)	Benzalkonium chloride (i.e. alkyl dimethyl benzyl ammonium chloride; approx. 80%) + isopropyl alcohol (2%) in water	Water	1 - 2%	2.00%
BiotinT	BT	C.T.S. S.r.l. (Altavilla Vicentina, Italy)	N-octyl-isothiazolinone (7.0-10.0%) + didecyl-dimethyl ammonium chloride (40.0-60.0%%) + formic acid (2.0-2.5%) + isopropyl alcohol (15.0-20.0%)	Water	1 - 3 %	2.00%
White Spirit	WS	Sinopia S.A.S. (Torino, Italy)	-	-	-	-
BiotinR	BR	C.T.S. S.r.l.	N-octyl-isothiazolinone (3-5%) + 3-iodoprop-2-ynyl N-butylcarbamate (10-25%) in diethylene glycol butyl ether	White Spirit (100%)	3 - 5%	3.00%
Acetone (≥99.9% for HPLC)	AC	Sigma-Aldrich (St. Luis, MO, USA)	-		-	-
Lichenicida 464	LI	Bresciani S.r.l.	4,5-Dichloro-2-octyl-4-isothiazolin-3-one (25.0-<40.0%) + 3-Iodo-2-propynyl N-butylcarbamate (12.5-<15.0%) + 2-Octyl-4-isothiazolin-3-one (0.06-<0.10%) + benzyl alcohol (40.0-<60.0%)	Acetone (100%)	1 - 2%	2.00%
Usnic Acid	UA	Sigma-Aldrich	Usnic acid [i.e. 2,6-Diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2H,9bH)-dione; powder]	Acetone (1%)	-	0.0005%

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Table 2 - Summary of the Generalized Linear Models examining the effects of predictors on F_V/F_M, written as F_V/F_M~Biocide × Application method × Site × Time. *, P<0.05; **, P<0.01; ***, P<0.001.

Predictor	*P. muralis*					*V. nigrescens*				
	Estimate	**Standard error**	**t value**	**P value**		**Estimate**	**Standard error**	**t value**	**P value**	
(Intercept)	5.02E-01	2.58E-02	19.443	<2E-16	***	5.91E-01	2.57E-02	22.987	<2E-16	***
Biocide-AC	5.11E-02	3.53E-02	1.449	0.1481		-4.15E-02	3.54E-02	-1.171	0.24228	
Biocide-BR	5.69E-02	3.53E-02	1.612	0.1076		**-6.97E-02**	**3.54E-02**	**-1.968**	**0.04958**	*
Biocide-BT	2.87E-02	3.53E-02	0.814	0.4159		-3.59E-02	3.54E-02	-1.012	0.31187	
Biocide-DN	3.68E-02	3.48E-02	1.056	0.2914		-3.61E-02	3.54E-02	-1.018	0.30907	
Biocide-GL	4.87E-02	3.53E-02	1.381	0.168		-4.53E-02	3.54E-02	-1.28	0.20108	
Biocide-LI	5.01E-02	3.53E-02	1.419	0.1566		-5.82E-02	3.54E-02	-1.644	0.1007	
Biocide-PV	-5.92E-05	3.53E-02	-0.002	0.9987		-4.72E-02	3.54E-02	-1.332	0.18346	
Biocide-UA	3.57E-02	3.53E-02	1.011	0.3125		-3.65E-02	3.54E-02	-1.031	0.30322	
Biocide-WS	3.65E-02	3.53E-02	1.033	0.3019		**-9.94E-02**	**3.54E-02**	**-2.806**	**0.00521**	**
Time T1	6.66E-02	3.48E-02	1.915	0.056	.	9.99E-03	3.45E-02	0.29	0.77214	
Time T20	1.81E-02	3.48E-02	0.52	0.6035		-4.36E-02	3.54E-02	-1.23	0.21914	
Application-Poultice	**-5.95E-02**	**9.18E-03**	**-6.487**	**2.07E-10**	***	**-7.32E-02**	**9.35E-03**	**-7.834**	**2.76E-14**	***
Site A (Industria)	**8.65E-02**	**1.12E-02**	**7.724**	**5.92E-14**	***	7.57E-03	1.14E-02	0.662	0.50804	
Site B (Luni)	-7.84E-03	1.13E-02	-0.694	0.4883		-1.77E-02	1.15E-02	-1.543	0.12355	
Biocide AC : Time T1	**-4.61E-01**	**4.99E-02**	**-9.238**	**<2E-16**	***	**-4.41E-01**	**5.01E-02**	**-8.815**	**<2E-16**	***
Biocide BR : Time T1	**-5.99E-01**	**4.99E-02**	**-12.001**	**<2E-16**	***	**-4.19E-01**	**5.01E-02**	**-8.366**	**5.70E-16**	***
Biocide BT : Time T1	**-2.76E-01**	**4.99E-02**	**-5.533**	**5.02E-08**	***	**-2.76E-01**	**5.01E-02**	**-5.516**	**5.51E-08**	***
Biocide DN : Time T1	**-1.97E-01**	**4.92E-02**	**-4.004**	**7.16E-05**	***	**-1.36E-01**	**5.01E-02**	**-2.724**	**0.00666**	**
Biocide GL : Time T1	-9.75E-03	4.99E-02	-0.195	0.8451		-2.34E-02	5.01E-02	-0.467	0.64043	
Biocide LI : Time T1	**-5.92E-01**	**4.99E-02**	**-11.863**	**<2E-16**	***	**-4.29E-01**	**5.01E-02**	**-8.557**	**<2E-16**	***
Biocide PV : Time T1	**-3.35E-01**	**4.99E-02**	**-6.715**	**4.98E-11**	***	**-3.05E-01**	**5.01E-02**	**-6.091**	**2.21E-09**	***
Biocide UA : Time T1	2.04E-02	4.99E-02	0.408	0.6831		1.28E-02	5.01E-02	0.256	0.79834	
Biocide WS : Time T1	**-5.08E-01**	**4.99E-02**	**-10.186**	**<2E-16**	***	**-2.70E-01**	**5.01E-02**	**-5.4**	**1.03E-07**	***
Biocide AC : Time T20	**-3.32E-01**	**4.99E-02**	**-6.66**	**7.07E-11**	***	**-3.60E-01**	**5.07E-02**	**-7.093**	**4.39E-12**	***
Biocide BR : Time T20	**-5.08E-01**	**4.99E-02**	**-10.195**	**<2E-16**	***	**-2.79E-01**	**5.07E-02**	**-5.496**	**6.14E-08**	***
Biocide BT : Time T20	**-2.36E-01**	**4.99E-02**	**-4.741**	**2.76E-06**	***	**-2.61E-01**	**5.07E-02**	**-5.141**	**3.89E-07**	***
Biocide DN : Time T20	**-2.56E-01**	**4.89E-02**	**-5.238**	**2.38E-07**	***	**-2.61E-01**	**5.07E-02**	**-5.137**	**3.98E-07**	***
Biocide GL : Time T20	**-1.12E-01**	**4.99E-02**	**-2.239**	**0.0256**	*	2.28E-02	5.07E-02	0.449	0.65339	
Biocide LI : Time T20	**-4.93E-01**	**4.99E-02**	**-9.893**	**<2E-16**	***	**-3.79E-01**	**5.07E-02**	**-7.466**	**3.59E-13**	***
Biocide PV : Time T20	**-3.37E-01**	**4.99E-02**	**-6.748**	**4.06E-11**	***	**-3.14E-01**	**5.07E-02**	**-6.181**	**1.30E-09**	***
Biocide UA : Time T20	-3.59E-02	4.99E-02	-0.719	0.4724		4.52E-03	5.07E-02	0.089	0.929	
Biocide WS : Time T20	**-2.77E-01**	**4.99E-02**	**-5.557**	**4.41E-08**	***	**-1.54E-01**	**5.07E-02**	**-3.043**	**0.00246**	**

Table 3 - Synoptic comparison of biocide efficacy at T20 against the photobionts of *P. muralis* and *V.nigrescens* at the three sites. For each species, biocide treatments which determined devitalization (†,*, or †*) in all the heritage sites are marked in bold. Biocide abbreviations are reported in Table 1.

	Brush			Poultice		
P. muralis	Site A (Industria)	Site B (Luni)	Site C (Boboli)	Site A (Industria)	Site B (Luni)	Site C (Boboli)
W	**=**	**=**	**=**	**=**	**=**	**=**
GL	**=**	-	-	**=**	**=**	-
DN	**=**	-	**=**	*	†	-
PV	*	-	-	*****	**†**	**†***
BT	**=**	-	**=**	*****	**†***	*****
WS	-	-	**=**	-	†*	*
BR	**†***	**†***	*****	**†***	**†***	**†***
AC	*	**=**	-	**=**	†*	*
LI	**†***	**†***	*****	**†***	**†***	*****
UA	**=**	-	**=**	-	-	-
V. nigrescens						
W	**=**	**=**	**=**	**=**	-	**=**
GL	**=**	**=**	-	**=**	**=**	-
DN	-	†	-	†*	-	-
PV	†	**=**	**=**	**†***	**†**	*****
BT	*	**=**	**=**	†*	†	-
WS	**=**	**=**	**=**	**†***	**†**	*****
BR	-	**=**	*	**=**	†	†*
AC	†*	†	**=**	†*	†	-
LI	**†***	**†**	*****	**†**	**†**	*****
UA	**=**	**=**	-	**=**	**=**	**=**

=, F_V/F_M did not significantly decrease with respect to T0, and F_0 decreased with respect to T0 < 80%

-, F_V/F_M significantly decreased with respect to T0, but it was not significantly lower than the threshold fixed at 0.15, and F_0 decreased < 80%

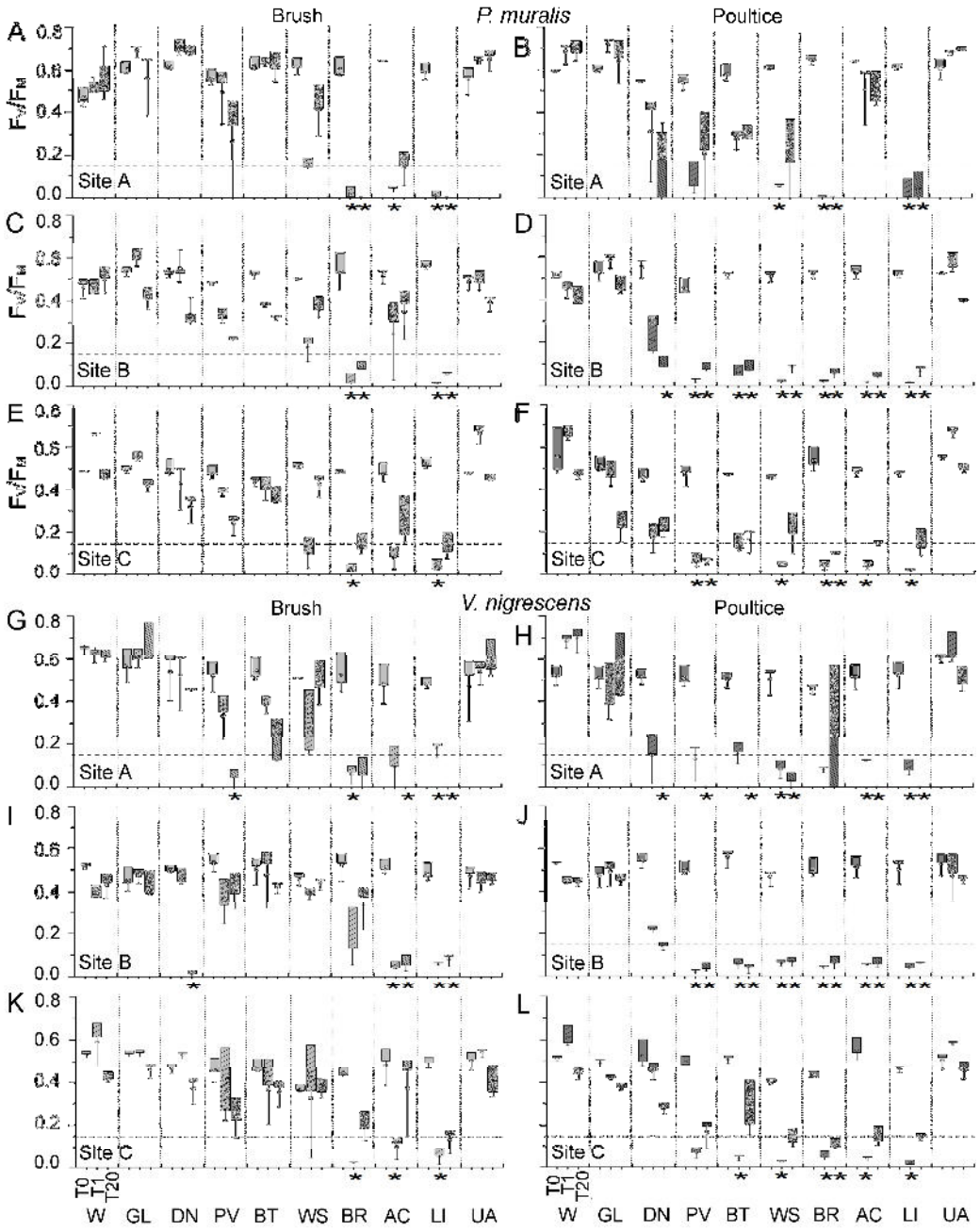
†, F_V/F_M significantly decreased with respect to T0 and it was significantly lower than 0.15, but F_0 decrease < 80%

*, F_0 decreased > 80%, but F_V/F_M was not significantly lower than 0.15

†*, F_V/F_M was significantly lower than 0.15, and F_0 decreased > 80%

Figure captions

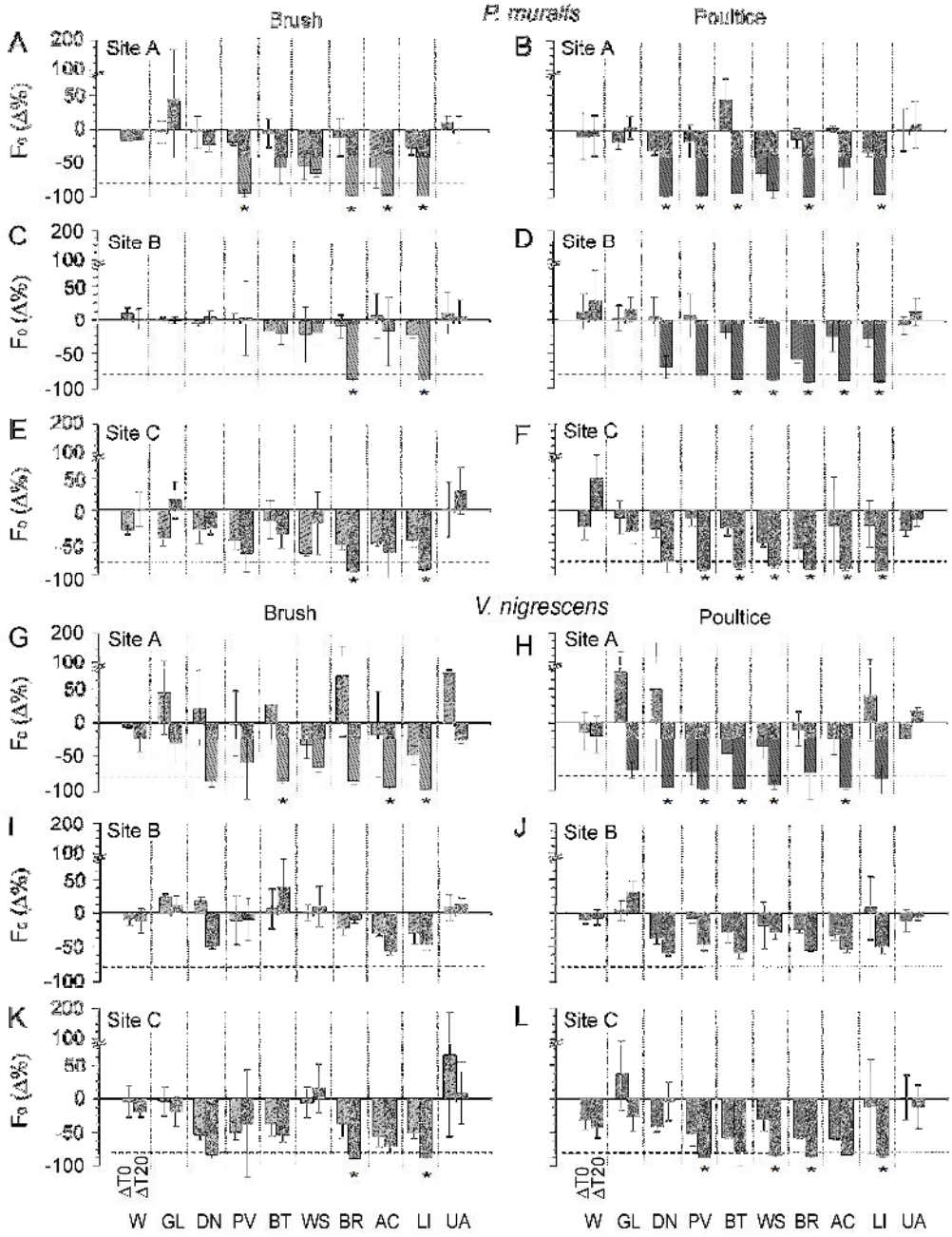
Fig. 1 - Maximum quantum efficiency of Photosystem II photochemistry (F_V/F_M) in thalli of *Protoparmeliopsis muralis* (A-F) and *Verrucaria nigrescens* (G-L) measured *in situ* (Site A, Industria; site B, Luni; site C, Boboli) before (T0), and 1 (T1) and 20 (T20) days after the biocide application with brush or using a cellulose poultice. Biocide abbreviations are reported in Table 1. For each case study (biocide × application method × site), F_V/F_M values (mean ±SD) which are significantly lower than a threshold fixed at 0.15 (horizontal dashed line) are marked (*; ANOVA, t-test; $P<0.05$). Overview tables of measures on *P. muralis* and *V. nigrescens*, including a statistical comparison for each study case of F_V/F_M values at T0, T1 and T20, are reported in Supplementary Materials S1 and S2, respectively.


Brush
P. muralis
Poultice
A
B
C
D
E
F
Site A
Site B
Site C
Fv/FM
V. nigrescens
Brush
Poultice
G
H
I
J
K
L
T0
T1
T20
W
GL
DN
PV
BT
WS
BR
AC
LI
UA

659

660

Fig. 2 - Variation of F_0 values (Δ% at T1 and T20 with respect to T0, i.e. 1 and 20 days after the biocide application with brush or using a cellulose poultice) in thalli of *P. muralis* (A-F) and *V. nigrescens* (G-L) examined in site A, Industria, site B, Luni, and site C, Boboli. Biocide abbreviations are reported in Table 1. For each case study (biocide × application method × site), variations of F_0 (mean ±SD) which are significantly lower than -80% (horizontal dashed line) are marked (*; ANOVA, t-test; $P<0.05$).

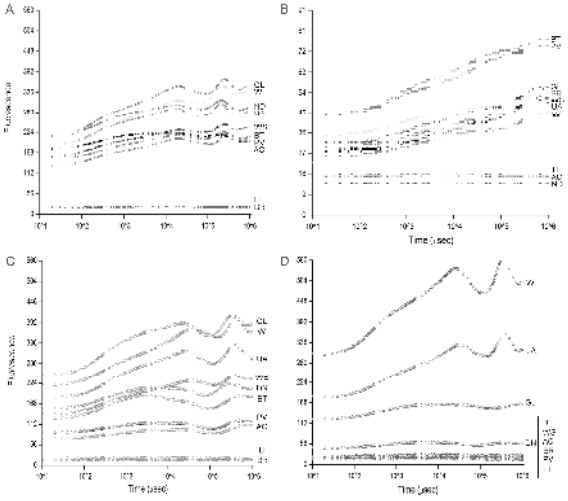

P. muralis
Brush
Poultice
A
Site A
B
Site A
C
Site B
D
Site B
E
Site C
F
Site C
V. nigrescens
Brush
Poultice
G
Site A
H
Site A
I
Site B
J
Site B
K
Site C
L
Site C
Fo (Δ%)
200
100
50
0
-50
-100
ΔT0
ΔT20
W GL DN PV BT WS BR AC LI UA

667

668

669 Fig. 3 - OJIP fluorescence transients at T20 (exemplifying set) of *P. muralis* (A) and *V. nigrescens*
670 (B) in site B (Luni) after the biocide application with brush, and of *P. muralis* in site C (Boboli)
671 after the biocide application with brush (C) or using a cellulose poultice (D). Each transient is the
672 average of the data obtained for the different replicates (5 measures for 3 thalli) of each case study.
673 Biocide abbreviations are reported in Table 1.

674



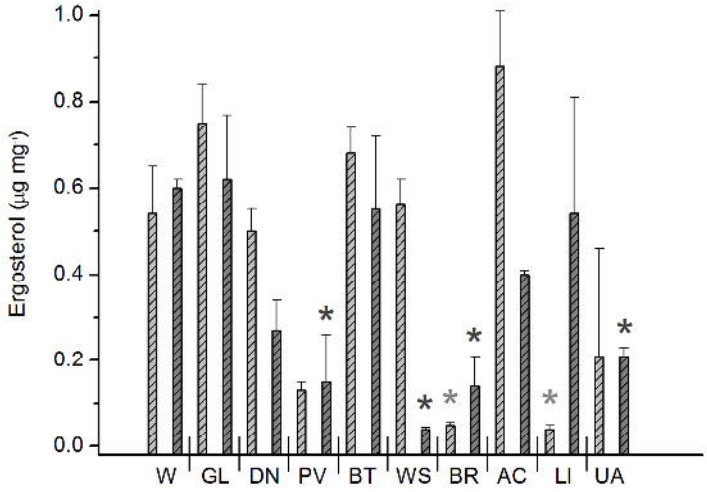
675

676

677

678 Fig. 4 – Ergosterol content in thalli of *P. muralis* in site C (Boboli) 20 days after the biocide
679 application (T20) by brush (light grey columns) or with cellulose poultice (dark grey columns).
680 Data are expressed as mean value ± SD. For each application method, significant decreases in
681 ergosterol following the biocide treatments, with respect to contents in thalli sprayed with water
682 only, are indicated (*, ANOVA, Tukey's test; $P<0.05$). Biocide abbreviations are reported in Table
683 1.

684



685