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Efficacy of the environmentally sustainable microwave heating compared to biocide applications in the devitalization of phototrophic communities colonizing rock engravings of Valle Camonica, UNESCO world heritage site, Italy

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Abstract

The devitalization of lithobionts prior to their removal from engraved rocks is a common conservation practice periodically undertaken in rock art sites. In this study, we assessed *in situ* the efficacy of three traditional biocides and of an innovative microwave heating system, and compared different application protocols to devitalize foliose and crustose lichens and a cyanobacteria-dominated biofilm on the rock engravings of Valle Camonica (UNESCO site n.94, Italy). The analysis of their vitality and stress responses by monitoring chlorophyll *a* fluorescence parameters (F_v/F_m, F_0, OJIP transient) showed that the common application of biocides by brush is rather ineffective, particularly in the case of the resistant crustose lichens. The heating of rock surfaces to 70°C for a few minutes by the microwave system caused devitalization of lithobionts to a similar extent as the biocide application with cellulose poultice, which, however, introduced high amounts of chemicals in the environment. The microwave irradiation overcame any lithobiontic stress resistance and avoided useless or excessive spread of biocides, appearing a promising sustainable approach for the parallel conservation of rock art and its surrounding natural environment.

Keywords:

chlorophyll *a* fluorescence, environmentally safe art restoration, cyanobacteria, lichens, microwave, biocide

1. Introduction

Open-air rock art sites are exposed to human actions and natural processes which can affect their preservation, i.e. perpetuation of heritage asset, and conservation, i.e. physical lifetime (Darvill and Batarda Fernandes 2014). Natural threats of stone cultural heritage include lithobiontic (i.e. rock dwelling) microorganisms, which generally affect the aesthetic appearance and historic value and put at risk the conservation of artworks because of their biodeterioration potential (Pinna 2017; Favero-Longo and Viles 2020). In the case of engraved rocks, the spatial extension of physical and chemical interactions of lithobionts with the mineral substrate may be dimensionally similar to that of the heritage objects (e.g. rock paint or engraving) and should thus deserve special attention in management plans (Knight et al. 2004). The main public concern often relates to the aesthetic alteration caused by the lithobiontic covering which masks the appearance and alters the legibility of rock art surfaces to scholars and other visitors, thus affecting research activities and the tourism industry. Therefore, the removal of lithobiontic communities is a common periodic practice undertaken in many rock art sites. The simple mechanical removal of lithobionts from heritage surfaces mostly favors the persistence of live structures within the substrate and the spread of viable fragments thereof (Pinna 2017). Its effect in cleaning of rock art is generally only temporary (Tratebas 2004 with refs. therein). Treatments using biocides have thus been widely combined with mechanical cleaning, but only in a few cases the efficacy of the adopted protocols has been evaluated by experimental assays on the devitalization effects and supported by medium- and long-term monitoring programs of their impact on the surface stability and bioreceptivity (Tratebas 2004; Sanmartín et al. 2019). As the efficacy of biocide treatments against lithobionts is both species- and site-specific, biocides' assays need to be performed *in situ* and focused on the conservation threats of each site (Favero-Longo et al. 2017).

On the other hand, alternative and sustainable devitalization approaches are increasingly invoked to drastically reduce the use of chemicals, their potential interference with the rock substrates and their threats to humans and the whole biosphere (UNESCO 2008; Cappitelli et al. 2020; Sanmartín et al. 2020). Recently, physical approaches have been experimented on rock art and other heritage surfaces by applying methods such as temperature shifts, electromagnetic radiations (e.g. gamma rays, UV, microwaves), and laser (Sanz et al. 2015; Pinna 2017 with refs. therein; Pozo-Antonio and Sanmartín 2018; Pozo-Antonio et al. 2019). Regarding temperature shifts, the heating of rock surfaces to 55-60°C, easily reached under direct sun radiation, is sufficient to devitalize lichens and other biodeteriogens within few hours if they are artificially kept fully hydrated (Tretiach et al. 2012; Bertuzzi et al. 2013). However, the success of this approach strongly depends on the available sun radiation and, thus, on meteorological conditions, in a way that is hardly compatible with the planning of restoration interventions in temperate countries. The same approach was recently implemented shifting the energy input from the sun radiation to a microwave heating system (MW) suitable for usage in the field (Riminesi and Olmi 2016).

Microwave heating has already been applied to kill various targets, including insects and microorganisms threatening stored food materials and, more recently, heritage artefacts (Macana and Baik 2018; Cappitelli et al. 2020; Soni et al. 2020). Regarding the stone, a preliminary in vitro investigation highlighted the devitalization effect of MW on black fungi (Cuzman et al. 2013). Its potential has been also confirmed in the field on the easy geometries of tombstones (Mascalchi et al. 2015, 2020). However, the efficacy of microwaves against components of phototrophic communities colonizing rock art (lichens and cyanobacteria) needs to be proven. Moreover,

information is lacking on the application of MW on the irregular shapes of natural outcrops hosting rock art.

This work aimed to assess *in situ* the efficacy of different approaches, including traditional chemicals and the innovative MW, to devitalize lithobionts prior to their removal from engraved sandstone outcrops in the Rock Engravings National Park of Naquane in Valle Camonica, part of the UNESCO world heritage site n. 94 (Italy). Comparative assays encompassed (i) three commercial biocides and MW, (ii) different application methods, and (iii) three biological targets widespread in the site, namely a cyanobacteria-dominated phototrophic biofilm, crustose and foliose lichens. We tested the hypothesis that the same effects of traditional biocides may be obtained with the environmentally safe MW. The devitalization and the physiological resistance of lithobionts were evaluated measuring chlorophyll *a* fluorescence, an indicator of photosynthetic activity.

2. Materials & Methods

2.1. Study site and target species

Microwave and chemical treatments were performed on Rocks 30 and 31, respectively, of the Rock Engravings National Park of Naquane, in middle Valle Camonica [Capo di Ponte, Brescia, Italy: UTM ED50, N 5097692, E 604391; 475 m]. This intra-alpine area displays rainfall around 1000 mm yr^{-1} and air temperatures ranging from av. 2°C in winter to av. 21°C in summer (monitoring station n. 129 of ARPA Lombardia, Capo di Ponte, 342 m a.s.l., dataset 2003-2016). Petroglyphs are carved in sandstone outcrops of the Verrucano Lombardo Formation (Upper Permian; Brack et al. 2008), which are widely colonized by phototrophic biofilms and lichens. Coccoid (*Gloeocapsa* sp., *Chroococcus* sp.) and filamentous (*Scytonema* sp., *Stigonema* sp.) cyanobacteria are the main components of biofilms, also including green algae, primordia of lichen thalli and microcolonial black fungi. Foliose thalli of *Xanthoparmelia* [mostly *X. conspersa* (Ach.) Hale] and mesophytic crustose species [mostly *Circinaria caesiocinerea* (Malbr.) A. Nordin, Savić & Tibell, *Pertusaria flavicans* Lamy, *Rhizocarpon disporum* (Hepp) Müll. Arg., and *Rufoplaca* gr. *arenaria* (Pers.) Arup, Søchting & Frödén,] are the dominant species in the lichen communities (Favero-Longo and Matteucci 2022).

Documentation available on cleaning interventions in the Park (irweb.it), registered from early 1980s, does not mention Rocks 30 and 31. Thus they have been likely left uncleaned for not less than 40 years. Treatments were performed on 15-30 cm ×15-30 cm parcels on the phototrophic biofilm, and on selected mature thalli of the foliose lichen *Xanthoparmelia conspersa* and the crustose *Rufoplaca* gr. *arenaria* (Fig. S1a).

2.2. Biocide application

The three biocides were prepared following the manufacturer's instructions as follows: 2% Preventol RI80 ® (benzalkonium chloride, ~80%, as active principle; Lanxess, Köln, Germany) in water, 3% Biotin T ® (N-octyl-isothiazolinone, 7-10%, and didecyl-dimethyl ammonium chloride, 40-60%, as active principles; CTS, Altavilla Vicentina, Italy) in deionized water, and 3% Biotin R ® (N-octyl-isothiazolinone, 3-5%, and 3-iodo-2-propynyl butylcarbamate, 10-25%, as active principles; CTS) in white spirit. Professional restorers applied the biocides in April 2018 on the phototrophic biofilm and lichens pre-hydrated with sprayed water (Favero-Longo et al. 2020). The

application was carried out using a paintbrush (Fig. S1b) and a cellulose poultice (Fig. S1c). The poultice was removed using a small spatula after 4 h, and then the treated thalli and biofilms were gently washed with tap water. Deionized water only was also separately applied as negative control. Three replicates per target organism (*X. conspersa, R. arenaria*, phototrophic biofilm) per product (deionized water, DW; Preventol, PV; Biotin T, BT; Biotin R) per application method (brush, poultice) were examined [equivalent to a total of 72 assays].

2.3. Microwave application

A portable microwave system for localized surface and sub-surface treatment (Fig. 1a) was employed in this study (Riminesi and Olmi 2016). Its antenna, subsequently designated as 'applicator', consists of a truncated rectangular waveguide with a properly designed slot on the aperture. The geometry of the applicator allows the devitalization of lithobiontic microorganisms, growing on and beneath stone surfaces, by concentrating the microwave field distribution on a semi-ellipsoidal volume of the treated material, with an elliptical surface footprint 4cm × 3cm in size and a depth of approx. 1.5 cm (Riminesi and Olmi 2016). A key aspect of the method is that microwave radiation heats targets containing water, allowing the selective treatment of living cells with a higher water content than that of the materials hosting the organisms..

The treatment was performed in June 2019 with two application methods: the applicator placed in direct contact with the colonized rock (Fig. 1b) and on a layer of cellulose poultice moistened with water (Fig. S1c). In both cases, the rock surface and the lithobionts were previously moistened with sprayed water. Heating of the rock surface was monitored in real-time by a non-conductive fiber optical sensor (Luxtron 1000A/A with fluoroptic probe; Luxtron, Cuneo, Italy). The microwave application was stopped when the rock surface had reached the threshold of 70°C for 170±4 sec, which was recognized as the best microwave dose against other lithobionts (that is black fungi; Cuzman et al. 2013). The cellulose poultice was removed a few minutes after the microwave application and the surface was gently washed with tap water.

Three replicates per target organism per application method were examined [n = 18, equivalent to 3 target organisms × 2 application methods × 3 replicas].

2.4. Vitality measurements

Chlorophyll *a* fluorescence (Chl_aF) of phototrophic biofilms and lichens was quantified 4-6 hours before (T0), 24 hours (T1) and 40 (T40) days after the treatments. In particular, the monitoring of biocide effectiveness was performed on April 10th ($T0_B$, where "$_B$" stands for "Biocides"), April 11th ($T1_B$) and May 19th ($T40_B$) 2018, and that of microwave effectiveness on June 20th 2019 ($T0_M$, where "$_M$" stands for "Microwaves"), June 21st ($T1_M$) and July 30 ($T40_M$) 2019. Although the monitoring of biocide and microwave assays was not performed at the same time due to logistic constrains, the rather similar mild and rainy climate conditions of the study area in central-spring ($T0_B$-$T40_B$) and early-summer ($T0_M$-$T40_M$) periods (Fig. S2) were considered acceptable to analyze the treatment effects on similarly active lithobiontic targets. Nevertheless, any differences in the starting photosynthetic efficiency observed at $T0_B$ and $T0_M$ were taken into account in the evaluation of biofilm and lichen responses (sections 3.1 and 4.1).

Chl_aF was measured with a Handy-PEA fluorimeter (Hansatech Instruments Ltd., Norfolk, England; saturating light pulse of 1s, 1500 μmol $m^{-2}s^{-1}$, peak at 650 nm) on moistened thalli and biofilms, previously obscured with a black fabric for twenty minutes to allow dark-adaptation. All

measurements were collected early in the morning, before the stone heating by sun, except for those at $T40_B$, which were performed late in the morning, when air temperature was approx. 20°C, but the sun radiation had already started to warm the rock surface. At each time point, five measurements were performed on each thallus of *X. conspersa* and *R. arenaria*, and on the biofilm parcels. The maximum quantum yield of PSII (F_v/F_m, with $F_v=F_m-F_0$, F_0 and F_m = the minimum and maximum fluorescence, with all reaction centres open and closed, respectively), indicates the functionality of the photosynthetic process and, thus, the general level of phototrophic lithobionts' fitness (Tretiach et al. 2010, 2012). F_0, which is related to the chlorophyll *a* content (Sanmartín et al. 2019), was also monitored. The threshold 0.15 of F_v/F_m values and the decrease of F_0 >80% were considered as reference values for devitalized lichens, that is, when their metabolic recovery can be confidently ruled out (Favero-Longo et al. 2017 with refs. therein). Moreover, the OJIP transient was examined, that is the Chl_aF polyphasic curve from F_0 (O) to F_m (P), with two steps at 2 ms (J) and 30 ms (I). The very fast O to J phase (photochemical phase) is mostly due to the reduction of the primary acceptor quinone of PSII (Q_A), being indicative of antenna size and connectivity of PSII reaction centres (Stirbet and Govindjee 2011; Malaspina et al. 2015). The J to I and I to P rises (thermal phase) are associated to the reduction of the plastoquinone-pool centers and the electron flow through PSI, respectively (Stirbet and Govindjee 2011; Malaspina et al. 2015). Another inflection is sometimes observed at 300 μs (K-step) as a response to thermal stress, attributed to the inactivation of the oxygen-evolving complex (Strasser 1997; Stirbet et al. 2019). The study of the O(K)JIP curve thus contributes to understand the impact of stress factors on the structure and functioning of the photosynthetic apparatus.

As a complement of Handy-PEA measurements, the responses of lichens and the phototrophic biofilms observable with the naked eye were visually monitored and recorded using the digital camera of an iPhone 5. Moreover, lobes of *X. conspersa* thalli were cut with a lancet at T0, T1 and T40, cross-sectioned and observed using an epifluorescence microscope Nikon Eclipse 300 to obtain spatial information on the devitalization of the photobiont layer (Favero-Longo et al. 2017).

2.5. Statistics

A factorial ANOVA was used to detect significant differences in Chl_aF parameters (F_v/F_m, F_0) according to the following independent variables: type of treatment (DW, PV, BT, BR, MW), time point (T0, T1, T40), application method (brush/direct, cellulose poultice), and target lithobiont (*X. conspersa*, *R. arenaria*, phototrophic biofilm). For each treatment, significant differences in F_v/F_m and F_0 values at the different time points were analyzed by ANOVA with post-hoc Tukey's test ($P<0.05$ as significant). As for microwave treatments, the times needed to reach temperature thresholds fixed from 30°C to 80°C (at each 5°C interval) when the applicator was applied directly on the rock surface or with the cellulose poultice were compared by ANOVA with post-hoc t-test, and time intervals of the exposition to temperatures higher than thresholds fixed from 30°C to 80°C (at each 10°C interval) were compared for the different study cases (target organisms × application methods) by ANOVA with post-hoc Tukey's test. All these analyses were carried out using SYSTAT 10.2 (Systat Software Inc., San Jose, CA).

The shapes of OJIP curves were compared using the PEA Plus 1.12 software package (Hansatech Instruments Ltd., UK).

3. Results

3.1. Chl$_a$f of lichen thalli and the biofilm before treatments

Chl$_a$f of the lichen thalli and of the biofilm was measured before the treatments with biocides and microwaves in April ($T0_B$) and June ($T0_M$), respectively. Negative controls (deionized water treatments) were monitored between April ($T0_B$) and May ($T40_B$) in parallel with biocide treatments.

The F_v/F_m values (Fig. 2) of negative controls were uniform at the different time points ($T0_B$, $T1_B$, $T40_B$), but differed significantly between biological systems (*X. conspersa*, av. 0.68 > *R. arenaria*, av. 0.44 > cyanobacterial biofilm, av. 0.26). Similarly, F_0 was higher in the foliose lichen thalli (av. 85) than in the crustose ones (av. 35) and the phototrophic biofilm (av. 22) (Fig. 3). F_0 values of the lichens and the biofilm measured at $T0_M$ were generally higher than those measured at $T0_B$.

The fluorescence transient curves of untreated *X. conspersa* (negative controls, and $T0_B$ and $T0_M$ of the different assays; Fig. 4a and Fig. S3a) showed the typical OJIP shape, as expected for unstressed thalli. A remarkable increase of F_0 and F_M, and of the whole amplitude of the curve, was observed at $T0_M$ with respect to $T0_B$. *R. arenaria* (Fig. 4b and Fig. S4a) similarly showed the characteristic sequence of the OJIP steps at $T0_M$, while F_0, F_M and the amplitude of the curve were remarkably lower at $T0_B$. At this time, a first peak along the curve was observed at 300 μsec (K-step), less pronounced or absent at $T0_M$. At $T40_B$, the negative controls of both lichens showed an anticipation of the P phase, observed at 0.3 sec. rather than at 0.5 sec.

The fluorescent transient curves of the biofilm were of much lower amplitude than those of the foliose and crustose lichens (Fig. 4c and Fig. S5a). F_0 and F_M were higher at $T0_M$ than at $T0_B$. At this latter time, in particular, the amplitude of the IP phase was minimal, while it was better recognizable, in the series of negative controls, at $T40_B$. A K-step was observed at both $T0_B$ and $T0_M$.

3.2. Heating of the rock surfaces treated with microwave radiation

Real-time temperature monitoring of the surfaces treated with microwaves is summarized in Fig. 5. All the parcels were exposed to rather similar heating rates, with the rock surface temperature quickly increasing to 50°C (after 19±6 sec, mean±SD), 60°C (45±10 sec), and 70°C (104±21 sec) (Fig. 5a). All the parcels were exposed to a temperature higher than 50°C for approx. 240 sec and to a temperature equal or higher than 70°C for approx. 170 sec (Fig. 5b). The heating rate was slightly faster for the parcels covered with cellulose poultice (Fig. 5a). Therefore, the total irradiation time on these parcels was shorter than that of the parcels treated with the applicator in direct contact (particularly in the case of *R. arenaria*), and temperatures even briefly increased above 80°C.

3.3. Effects of biocides and microwave heating on Chl$_a$f

At T40, each lithobiont treated with chemicals or MW exhibited a modified appearance in comparison to T0 and the controls, observable with the naked eye. The thalli of *X. conspersa* were yellowed (Fig. 1d-e and Fig. S6), those of *R. arenaria* appeared crumpled (Fig. S7), and the phototrophic biofilm exhibited wide detachments (Fig. S8). These modifications occurred

regardless the treatment type and the application method. However, such homogeneity in the visible effects was not reflected by Chl_aF responses.

The factorial ANOVA showed that all the examined variables (type of treatment, time, target lithobiont, application method) significantly contributed to the devitalization effectiveness, as expressed by the F_v/F_m and F_0 parameters (Table S1). Both chemicals and MW devitalized the target lithobionts, but the efficacy strongly depended on the application method.

After the application of the biocides by brush, F_v/F_m values of *X. conspersa* and *R. arenaria* remarkably decreased at $T1_B$ (-65% to -95%, out of Preventol on *X. conspersa*), but at $T40_B$ they showed a significant recovery above the vitality threshold of 0.15 (Fig. 2).
Uniquely, brush application of Preventol (PV) and Biotin T (BT) zeroed the median F_v/F_m values of the phototrophic biofilm, which was instead generally unaffected by Biotin R (BR). F_0 values of *Xanthoparmelia* at $T40_B$ were much lower than those at $T0_B$ and $T1_B$, but they neither zeroed nor decreased more than 80% (Fig. 3). Such fluorimetric results were also confirmed by epifluorescence microscopy, displaying the persistence of residual, red autofluorescent viable cells in the lower part of the photobiont layer (Fig. S9). *R. arenaria* and the biofilm treated with PV and BT showed a considerable increase of F_0 values at $T1_B$ and a decrease at $T40_B$. Only the biofilm showed zeroed F_0 values after PV application.
On the contrary, both lichens and the biofilm were strongly affected by all the chemicals when applied by the cellulose poultice. In all cases (out of PV on *R. arenaria*), Fv/Fm values at $T40_B$ were much lower than 0.15, and in most cases the strong devitalization effect was already detected at $T1_B$. F_0 values of *X. conspersa* at $T40_B$ strongly decreased (>80%) being quite zeroed. Regarding *R. arenaria* and the biofilm, the relative increase of F_0 at $T1_B$ was mostly followed at $T40_B$ by a strong decrease (>80%) and/or by zeroing of median values (with the confirmed exception of PV on *R. arenaria*).

The microwave heating system also showed different efficacies when applied directly on the rock or with the interposed layer of cellulose poultice. The direct application was very efficient, with F_v/F_m values of all lithobionts decreasing below 0.15 since $T1_M$ and zeroing at $T40_M$ in the case of *X. conspersa* and the biofilm. The zeroing of F_0 values at $T40_M$ confirmed the result. By contrast, MW application on the surfaces covered by the cellulose poultice was only effective on *X. conspersa* (F_v/F_m <0.15 at $T1_M$ and $T40_M$; ΔF_0>80%). Both *R. arenaria* and the biofilm showed a low increase of F_0 at $T1_M$ and only a limited decrease of F_v/F_m and F_0 values at $T40_M$.

Fluorescent transient curves confirmed the importance of the application method on the efficacy of biocides (Figs. S3-S5) and microwave radiation (Fig. 4). The poultice application of biocides (Fig. S3) and the direct application of MW on *X. conspersa* (Fig. 4a) led to the flattening of the curve since $T1_{B,M}$ and to its zeroing at $T40_{B,M}$. The application by brush of BR similarly flattened the curve at $T1_B$, while a minimal amplitude, with the appearance of a K-step at 300 μsec, was still observed with BT; a slight rise of the OJ phase and a reduction of the IP amplitude were observed with PV (Fig.S3). For all the biocides, a minimum curve amplitude was still observed at $T40_B$, with the OJ and IP phases recognizable for BT >>BR > PV (Fig. S3). MW application with the interposed layer of cellulose poultice determined the increase of F_0, but flattened the curve, which definitely zeroed at $T40_M$ (Fig. 4a).

BR and BT poultices (Fig. S4) and MW direct application (Fig. 4b) on *R. arenaria* determined at $T0_{B,M}$ an increase of F_0 and the flattening of the curve, which was still flattened and zeroed at $T40_{B,M}$. PV poultice also flattened the curve at $T1_B$, without the increase of F_0, but it recovered some amplitude and the characteristic OJIP steps at $T40_B$, with a well-defined K-step at 300 μsec (Fig. S4). Biocides applied by brush (Fig. S4) and the MW indirect application (Fig. 4b) also resulted in a strong reduction of the curve amplitude, with a remarked K-step and the flattening of the IP phase (and for BT and PV the increase of F_0). However, all the thalli recovered the characteristic OJIP steps at $T40_{B,M}$, more remarkably for BT, MW > BR, PV.

Regarding the phototrophic biofilm, all the biocides caused an increase of F_0 and F_M at $T1_B$. Poultice applications were characterized by a complete curve flattening, while a minimal preservation of the OJ rise and of the K-step followed the brush treatment (Fig. S5). At $T40_B$, the fluorescent transient was flat and zeroed for PV treatments, flat for BT treatments (but with F_0 around 10), and flat and zeroed for the poultice application of BR, while the OJ rise was still observed with brush. With the MW direct application (Fig. 4c), F_0 and F_M decreased since $T1_{M,}$ and the fluorescent transient was flattened and zeroed at $T40_M$. The MW indirect treatment increased F_0 and F_M values at $T1_M$, with some curve amplitude remaining, that is a profile similar to those observed with biocide treatments. The OJ rise was still observable also at $T40_M$.
It is worth noting that both chemical and MW applications, independently of their devitalization efficacy, did not directly lead to the complete removal of targeted lithobionts, which instead required successive interventions with mechanical tools to gently detach both lichen thalli and the phototrophic biofilm, and thus achieve surface cleaning (Fig. 1f).

4. Discussion

The devitalization of lithobionts before their removal has long been recognized as a necessary step to assure the efficacy and durability of cleaning procedures on engraved rock surfaces (Tratebas 2004). Unfortunately, if a biocide treatment fails to devitalize the target lithobionts, the restoration would be ineffective and unjustifiably pollutive too, because of the useless spread of chemicals in the environment. This drawback may be particularly critical when using biocides containing benzalkonium chloride, which was suggested to serve as a nutrient to microorganisms due to its nitrogen content, thus favoring recolonization dynamics (Scheerer et al. 2009), and to promote the selection of resistant microbial strains (Martin-Sanchez et al. 2012; Kim et al. 2018).

This work showed that the application of biocides on rock art surfaces by brush -a commonly practiced devitalization procedure saving time and materials- can be ineffective on resistant lithobionts, as crustose lichens are (Table 1). Moreover, our results confirmed the hypothesis that the heating of rock surfaces to 70°C for a few minutes by a microwave system causes the same effective devitalization of the targeted lithobionts obtained with the cellulose poultice application of biocides. It is worth mentioning that this latter approach introduces in the substrate higher amounts of biocides than the brush application (Favero-Longo et al. 2020), with consequent environmental concern. Heating by microwave radiation does not leave any residual on the substrate. When exposed to the oscillating microwave field, the water molecules of both lithobionts and the stone move (ionic conduction) and rotate (dipolar rotation), with their frictions resulting in heat generation and increase of temperature. After removing the microwave applicator, the molecules stop moving and vibrating, and the temperature comes back rapidly, without leaving any residual

effect, by emitting blackbody radiation in IR range (9-12 μm) or dissipating their heat by conduction (Metaxas and Meredith 2011). Accordingly, microwave irradiation does not impact rock surfaces with temperature shifts similar to those used in pulsed laser irradiation, which instead may cause thermal stress and the melting of rock-forming minerals (De Cruz et al. 2014; Pozo-Antonio et al. 2019).

At present, the portable microwave system (MW) only allows the irradiation of small surfaces, as it is necessary to perform multiple adjacent applications of the 4 cm × 3 cm applicator, each taking approx. 5 minutes to reach and maintain 70°C for approx. 3.0 minutes. This means that the device takes approx. 6-7 hours to cover 1 m^2, a treatment rate unsuitable to cover the wide outcrops of rock art sites, but effective to treat lithobionts on small, engraved rock areas of peculiar interest. The MW instrumentation is being developed further to allow treatments of larger surfaces. Here we discuss the species-specific efficacy of physical and chemical devitalization treatments in relation to the different physiological responses of target lithobionts, addressing critical issues for the management of rock art and the surrounding natural environment. A first insight on the microwave effects on the photosynthetic efficiency of lichens and cyanobacteria is provided.

4.1. Photosynthetic efficiency of target lithobionts

Different values of F_0 (and F_v/F_m) for each species at $T0_B$ and $T0_M$ indicated a seasonality of the photosynthetic performance, which has been previously reported for saxicolous lichens of the Mediterranean region, showing a marked reduction of quantum efficiency in summer drought periods, followed by the recovery of their optimum fluorescence in autumn (Vivas et al. 2017). However, the present study showed that lower F_0 and F_v/F_m values at $T0_B$ than at $T0_M$ were measured in a humid week of a wet spring (as usual in Valle Camonica; see Gerosa et al. 2013; cumulative rain in Oct17-Jul18 in Fig. S2b). Higher F_0 values and higher amplitude of OJIP curves registered at $T0_M$ (cumulative rain in Oct18-Jul19 in Fig. S2b) may be thus better explained by a recovery of the photobiont populations in the vegetative season, which determined a higher chlorophyll content in the thalli (Baruffo and Tretiach 2007), rather than by the absence of drought stress.

Different F_0 and F_v/F_m values at $T0_{B,M}$ of *X. conspersa* and *R. arenaria* indicated the well-known interspecific variability of the lichen photosynthetic performances, with lower values reported more often in crustose than foliose species (Jensen et al. 2002). However, although foliose species may be expected to harbour more photobionts and thus contain higher chlorophyll contents than crustose ones, the photosynthetic performance seems to be not uniquely related to growth form, but also influenced by the substratum and the microenvironmental conditions (Nayaka et al. 2009). Accordingly, the direct contact of *R. arenaria* with the rock may regularly determine stressful conditions, revealed by the small K-step observed along the OJIP curve already at $T0_{B,M}$, and instead not detected in *X. conspersa*, in which the rhizinae are interposed between the rock and the thallus. In plants, the K-step has been related with a heating induced injury in the oxygen-evolving complex of PSII, determining an imbalance in the electron flow around P680 and the accumulation of oxidized reaction centers (Strasser 1997; Kalaji et al. 2016). Small K-steps as observed in this study for *R. arenaria*, even before any treatment, usually characterize highly stressed organisms (Marečková et al. 2019). Surprisingly, the K-step was not observed in *X. conspersa* even at $T40_B$,

when the measures were performed late in the morning of a sunny day, and the fluorescent transient was thus affected in the IP phase.

F_v/F_m values of the phototrophic biofilm at $T0_{B,M}$ (av. 0.26) were only slightly lower than maximum values reported for cyanobacteria *in vitro* (0.3-0.4; Gao et al. 2007). Such low values were obtained using the "apparent" F_0 and F_M values, that were measured not considering that cyanobacteria, unlike green algae and higher plants, use electron flows from PSII in both photosynthesis and respiration (Stirbet et al. 2019). Approaches to obtain more reliable F_v/F_m values (reviewed in Stirbet et al. 2019) were not followed here, as the devitalization was evaluated by adopting the same measuring conditions before and after both treatments. Similarly to lichens, the K-step at 300 μsec is a marker of heat stress in cyanobacteria (Zhang and Liu 2016; Kvíderová and Kumar 2020). It was observed in the measures of the phototrophic biofilm even before the devitalization treatments. Accordingly, the phototrophic biofilm and *R. arenaria*, more adherent to the rock, appeared regularly affected by heat stress, while *X. conspersa* was not.

4.2. Microwave heating and photosynthetic efficiency

The application of microwave heating directly on the rock surface moistened with sprayed water overcame the stress tolerance of all the lithobionts, with flattened fluorescent curves at $T1_M$, which did not show any recovery at $T40_M$ likely because of the high temperature of the irradiated surface. Indeed, 70°C could be tolerated by lichens when dry (Lange 1953), but not in the hydrated state, when their heat resistance ranged from 35° to 46°C (MacFarlane and Kerhaw 1980; Tretiach et al. 2012). The cyanobacteria-dominated phototrophic biofilm did not show the resistance demonstrated by certain epilithic green-algae, which survived even in the hydrated state to a heat-shock treatment at 60°C, as few unaffected cells re-established viable populations (Bertuzzi et al. 2017). MW application with the interposed poultice layer gave a better glimpse of the lithobiontic response to the heating stress. Together with the F_m decrease, the stress was indicated by F_0 increase in *X. conspersa* > phototrophic biofilm > *R. arenaria*, which may depend on the heat-induced presence of free chlorophyll and uncoupled antennas proteins (Strasser 1997). Remarkably, the lower F_0 increase showed by the lithobionts more adherent to the substrate, and likely more used to heat stress than *X. conspersa* (MacFarlane and Kershaw 1980), corresponded to their higher recovery at $F40_M$. Accordingly, in laboratory experiments simulating some microclimatic conditions of the warm Namib desert, the hydrated thallus of genus *Xanthoparmelia* (*X. walteri*) was still photosynthetically active at 55°C, while that of genus *Caloplaca* (*C. elegantissima*, sharing with *R. arenaria* the former genus *Caloplaca* s.l., before its recent subdivision based on molecular phylogeny; Arup et al. 2013), was inactive above 45°C; nevertheless, the latter species was the most widespread in the real desert, suggesting that its response implied some adaptation to this harsh environment (Lalley and Viles 2006; see next section). On the other hand, although the different lithobionts were exposed for the same time interval to 70°C and more, the different heating rate of each surface corresponded to a different total time of microwave irradiation. In particular, the duration was generally lower for the less effective poultice application, with the higher water availability likely accounting for the higher heating rates (Metaxas and Meredith 2011). The shortest irradiation interval and exposition to 40-60°C (but also the highest interval above 80°C) were observed for the poultice application on the more resistant *R. arenaria*. Accordingly, the time of exposition to temperatures around the limit of photosynthetic activity (approx. 50°C) may be

even more crucial in terms of treatment effectiveness than the high temperature (equal or above 70°C) at which lithobionts are metabolically inactive and thus more tolerant to stress (Lalley and Viles 2006).

4.3. Biocides and photosynthetic efficiency

The higher resistance of *R. arenaria* > phototrophic biofilm > *X. conspersa* was also confirmed by the results of biocide treatments, although there were some different patterns depending on the product. F_v/F_m<0.15 and the flattening and zeroing of the transient curve in *R. arenaria* were observed only after the application of BR and BT by poultice. These biocides contain isothiazolinones, which yield metabolic inhibition by targeting thiol-containing enzymes (Denyer and Stewart 1998). PV contains benzalkonium chloride (BZC), which damages biological membranes and causes cell lysis (Wessels and Ingmer 2013). Unlike the other two biocides, PV did not cause the devitalization of the crustose lichen. Although the transient curve was rather flat at $T1_B$, it recovered the OJIP shape at $T40_B$, including a small K-step, suggesting that the resistance to heating stress is suitable to provide resistance also to the biocidal action of the quaternary ammonium salt. This does not mean that quaternary ammonium salts did not affect the photobionts, because after the application by brush of PV and BT, which contain BZC and didecyl-dimethyl ammonium chloride, respectively, an increase of F_0 was observed at $T1_B$, possibly related to the occurrence of free chlorophyll caused by the membrane perturbation (Strasser 1997). However, this effect likely did not occur in the whole photobiont layer and was therefore ineffective. Similarly, another species of the genus *Caloplaca* s.l. (*Variospora aurantia*) was shown to preserve viable algae in some parts of the photobiont layer after the poultice application of BZC (1.5%) on carbonate blocks in a semi-arid environment, where lichens usually tolerate remarkable thermal and salt stresses (Matteucci et al. 2019). Differently, poultice applications of PV, and of the other two biocides, effectively devitalized the photobionts of *X. conspersa*, and also those of the crustose lichen *Verrucaria nigrescens* and of the placodioid *Protoparmeliopsis muralis*, which also frequently occur on heritage surfaces (Favero-Longo et al. 2017). The hypotheses that the *Trebouxia* photobionts involved in the symbiosis with the *Caloplaca* mycobionts are more stress resistant than those of *Xanthoparmelia* and others, or that the *Caloplaca* mycobionts confer more protection to their photobionts, allowing their resistance, appear worth to be investigated. Several species of genus *Caloplaca* s.l. display fungal and algal stacks as an adaptation to strong light radiation (Vondrák and Kubásek 2013), which may also confer tolerance to other stress factors.

F_0 increased at $T1_B$ when PV and BT were applied by brush, but not when applied by poultice. The result suggests that its initial rapid increase, caused by membrane damages, was still detectable the day after the application of a low amount of biocide, while it was followed by a quick decrease due to the more abundant amount of biocide. By contrast, F_0 increased only with the poultice application of BR, and it might be caused by the effect of white spirit solvent, as the same was observed when it was applied alone as control (not shown).

The biocide application by brush on both lichens was always followed by the recovery of Fv/Fm>0.15 and of the OJIP shape of the transient curve, more remarkable for *R. arenaria*, indicating the poor suitability of this method on potentially resistant crustose species such as those of genus *Caloplaca s.l.* By contrast, some higher efficacy was observed when PV and BT were

applied by brush on the cyanobacteria-dominated biofilm. This result is of remarkable interest as these lithobiontic communities widely covered the surfaces, and it is therefore difficult (and not environmentally safe) to plan a poultice application at the scale of the whole outcrops. The result is partially explained by the different hydrophilicity displayed by biofilms depending on the composition of their extracellular polymeric substances (EPS; Sanmartín et al. 2020), which in the examined case likely did not prevent the absorption of the water-dissolved quaternary ammonium salts. The cyanobacterial cell membranes, not protected by the mycobiont as lichen green algae are, were eventually damaged. Accordingly, a strong F_0 increase was observed at $T1_B$, after both brush and poultice applications, suggesting again the presence of free chlorophyll (Strasser 1997). This finding agrees with the positive results of PV applied on cyanobacteria on carbonate rocks, causing the complete disorganization of the prokaryotic cells (Ascaso et al. 2002), and with the significant decrease of chlorophyll *a* observed in planktonic cyanobacterial cultures (*Nostoc* sp.) treated with BT (Sanmartín et al. 2015). Oppositely, in this study, the phototrophic biofilm showed a better resistance to BR application by brush than that of BT, contrasting with previous findings about its treatment on carbonate substrates (de los Ríos et al. 2012). This result may depend on the different substrates and/or a diverse composition and hydrophilicity of the EPS, which may together influence the biocide availability and absorption (Favero-Longo et al. 2020; Sanmartín et al. 2020).

It is finally worth noting that all these different patterns of resistance or sensitivity to the various treatments were detected and quantified with the fluorimetric measures, which are confirmed as a crucial tool to assess the efficacy of devitalization protocols against phototrophic lithobionts, and validate their adoption in restoration interventions (Tretiach et al. 2010). By contrast, the sole observation of treated lithobionts with the naked eye may reveal a similar appearance for devitalized and (partially) live thalli and biofilms and it is thus not a reliable feedback to select effective strategies for biodeterioration control.

5. Conclusions

This work demonstrated that microwave heating represents an effective and sustainable method to devitalize cyanobacterial biofilms, crustose and foliose lichens that grow on the rock engravings of Valle Camonica, yielding the same successful devitalization obtained by an abundant biocide application, but avoiding any dispersal of toxic residues in the environment.

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the work in progress to the visitors of the Rock Engravings National Park. In this way the community is made aware of the management efforts for the conservation of the heritage site inscribed in the WHL and the environment.

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685 **Figure captions**

686 Fig. 1. Microwave treatment on Rock 30 (Rock Engravings National Park of Naquane, Valle
687 Camonica, Italy). (a) the portable microwave system; (b) microwave applicator on a parcel (30 × 30
688 cm) with *Rufoplaca arenaria* (*); (c) microwave application with an interposed layer of cellulose
689 poultice; (d-f) a surface with *Xanthoparmelia conspersa* before ($T0_M$, d) and 40 days after ($T40_M$; e)
690 the microwave treatment with the applicator in direct contact with the rock, and after the successive
691 cleaning using mechanical tools (f).

692 Fig. 2. Maximum quantum yield of PSII (F_v/F_m) in *Xanthoparmelia conspersa* (a), *Rufoplaca*
693 *arenaria* (b) and the phototrophic biofilm (c) 4-6 hours before (T0) and 24 hours (T1) and 40 days
694 (T40) after the treatment with deionized water only (DW), Preventol (PV), Biotin T (BT), Biotin R
695 (BR), and microwaves (MW), applied directly (brush or direct MW contact; light box plots) or with
696 the cellulose poultice (dark box plots). For each case study (biocidal approach × application method
697 × species), box plots not sharing any capital letter (A, B, C) differ significantly ($P<0.05$). F_v/F_m
698 values that significantly decreased below the threshold fixed at 0.15 ($P<0.05$), indicative of
699 devitalization, are marked (*).

700 Fig. 3. F_0 values *Xanthoparmelia conspersa* (a), *Rufoplaca arenaria* (b) and the phototrophic
701 biofilm (c) 4-6 hours before (T0) and 24 hours (T1) and 40 days (T40) after the treatment with
702 deionized water only (DW), PV, BT, BR, and MW, applied directly (brush or direct MW contact;
703 light columns) or with the cellulose poultice (dark columns). For each case study (biocidal approach
704 × application method × species), box plots not sharing any capital letter (A, B, C) differ
705 significantly ($P<0.05$). F_0 values that decreased at T1 and T40 more than 80% with respect to T0
706 ($P<0.05$) are marked (*).

707 Fig. 4. OJIP transients of *Xanthoparmelia conspersa* (a), *Rufoplaca arenaria* (b) and the
708 phototrophic biofilm (c) before ($T0_M$, filled quadrats), and 24 hours ($T1_M$, crossed quadrats) and 40
709 days ($T40_M$, empty quadrats) after microwave radiation applied directly (black symbols) or with an
710 interposed layer of cellulose poultice (red symbols). Time is expressed as seconds; fluorescence is
711 expressed as arbitrary units.

712 Fig. 5. Real-time temperature monitoring of surfaces treated with microwaves. (a) times (av ± SD)
713 needed to reach temperature thresholds (from 30° to 80°C) after the beginning of microwave
714 application; (b) time intervals (av. ± SD) in which the rock surfaces colonized by *Xanthoparmelia*
715 *conspersa* (X), *Rufoplaca arenaria* (R) and the phototrophic biofilm (P) were exposed to
716 temperatures higher than thresholds ranging from 30°C to 80°C. The treatment was carried out with
717 the microwave applicator positioned directly on the rock surface (black symbols, a, black columns,
718 b) or with an interposed layer of cellulose poultice (red symbols, a; red columns, b). For each
719 temperature threshold, couples of points marked with an asterisk (a) and columns not sharing any
720 capital letter (b) significantly differ ($P<0.05$).

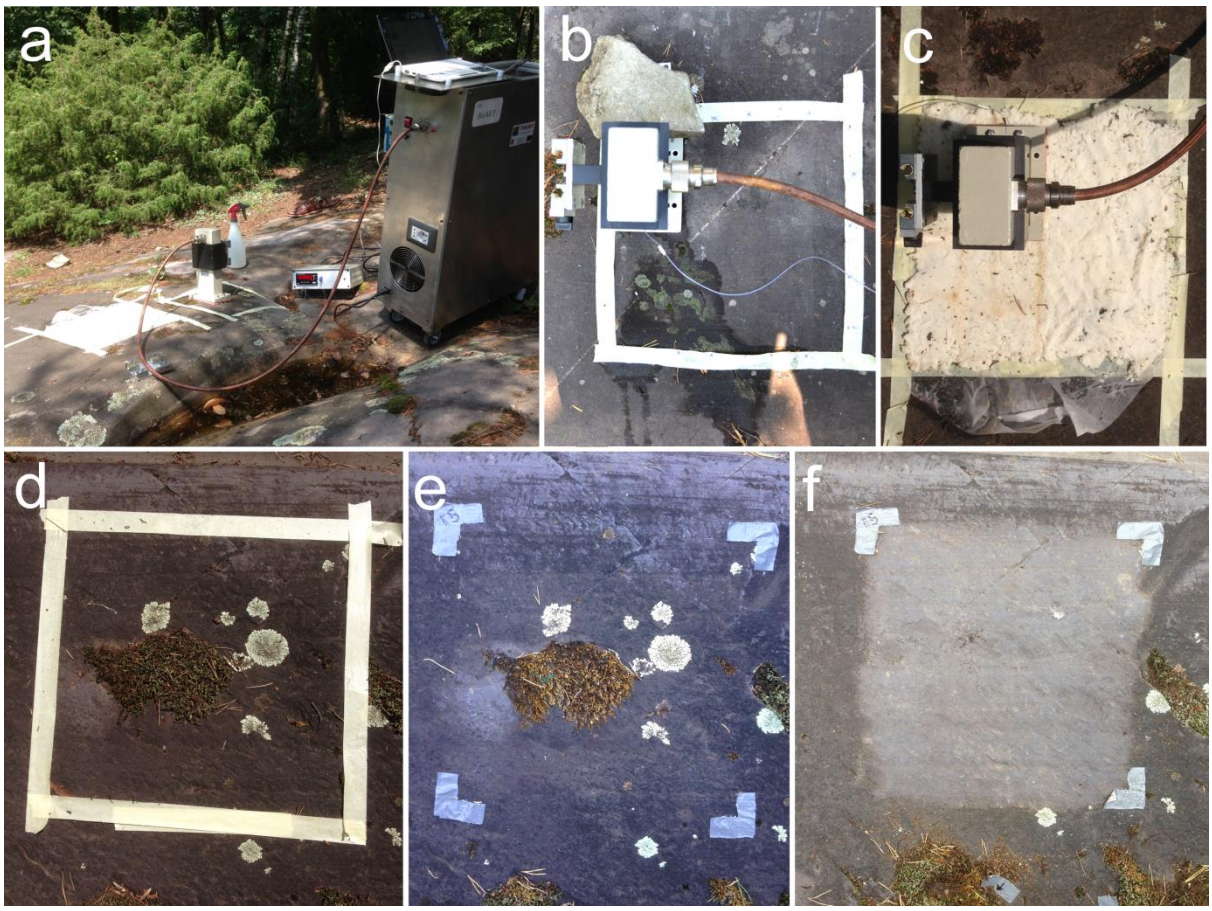
721

722 Table 1. Synoptic comparison of the treatment effectiveness against the foliose lichen
723 *Xanthoparmelia conspersa*, the crustose lichen *Rufoplaca arenaria* and the cyanobacterial
724 phototrophic biofilm, evaluated after the application of biocides (Preventol, PV; Biotin T, BT;
725 Biotin R, BR) and microwaves (MW) (at T40 with respect to T0).

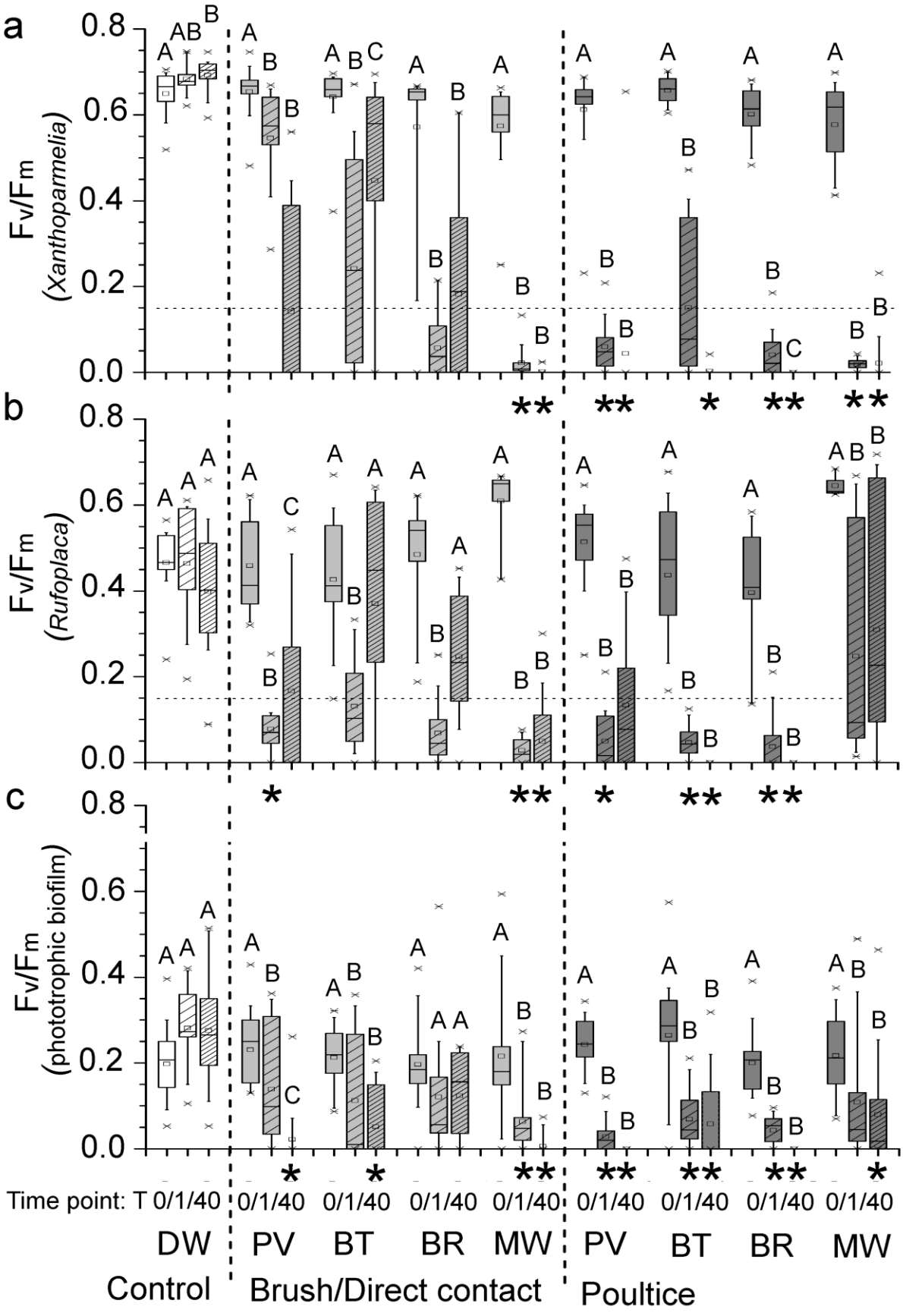
	Foliose lichen	Crustose lichen	Photo-trophic biofilm	Foliose lichen	Crustose lichen	Photo-trophic biofilm
	Application by brush			Application with cellulose poultice		
PV	-	-	†	†*	-	†*
BT	-	=	†	†*	†	†
BR	-	=	=	†*	†*	†*
	Direct application			Interposed cellulose poultice		
MW	†*	†*	†*	†*	-	†

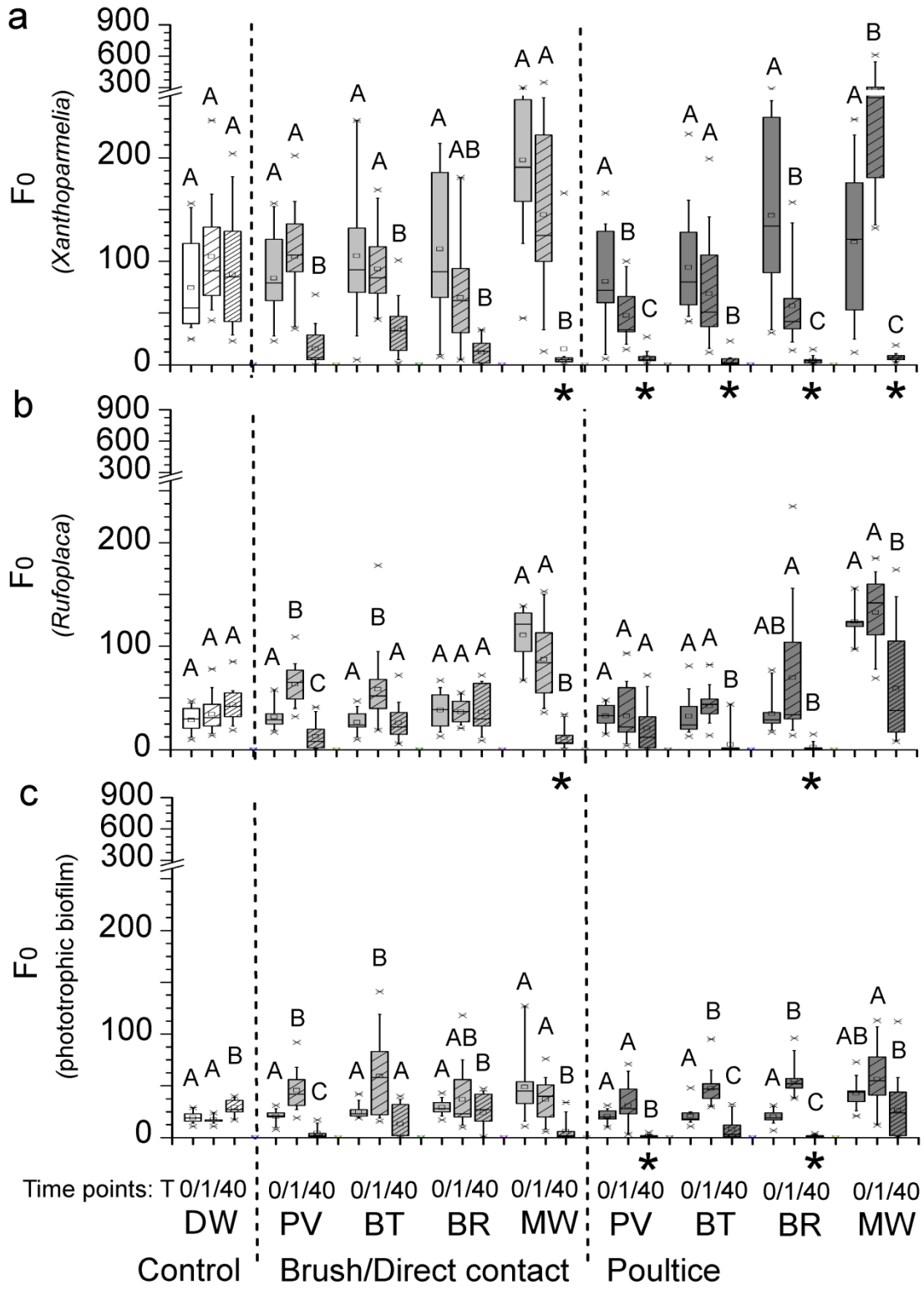
726 =, no significant decrease of F_v/F_m, and F_0 decrease lower than -80%
727 -, significant decrease of F_v/F_m, but not below the 0.15 threshold, and F_0 decrease lower than -80%
728 †, significant decrease of F_v/F_m, but not below the 0.15 threshold, and F_0 decrease higher than -80%
729 †*, significant decrease of F_v/F_m below 0.15, and F_0 decrease higher than -80%
730

731

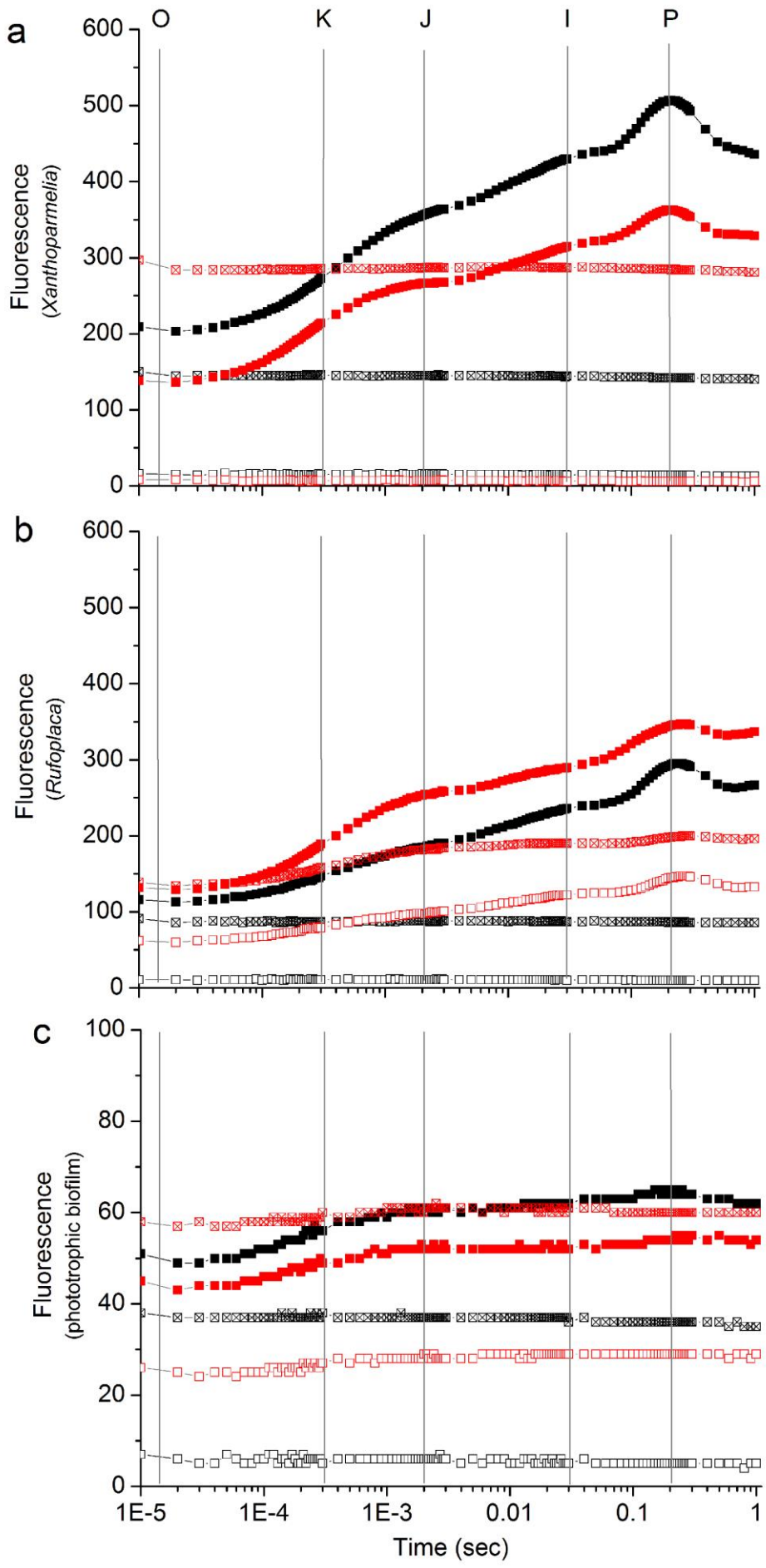


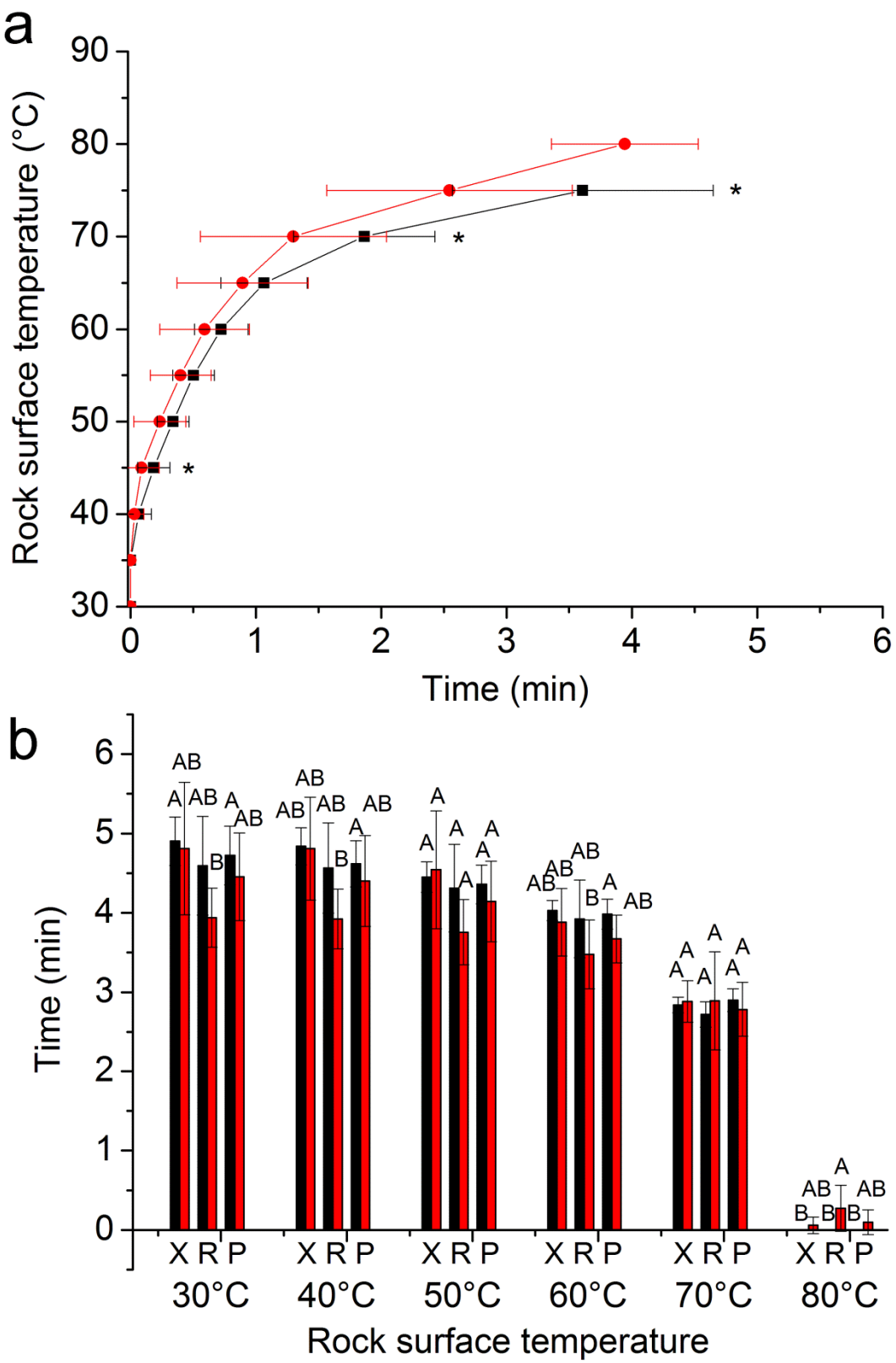
732


a
b
c
Fv/Fm
(Xanthopamelia)
(Rufoplaca)
(phototrophic biofilm)
0.0
0.2
0.4
0.6
0.8
Time point: T 0/1/40
0/1/40
DW
PV
BT
BR
MW
Control
Brush/Direct contact
Poultice


a
b
c
F0
(Xanthoparmelia)
(Rufoplaca)
(phototrophic biofilm)
900
600
300
200
100
0
Time points: T 0/1/40 0/1/40 0/1/40 0/1/40 0/1/40 0/1/40 0/1/40 0/1/40 0/1/40
DW PV BT BR MW PV BT BR MW
Control
Brush/Direct contact
Poultice

734


a
O
K
J
I
P
Fluorescence
(Xanthoparmelia)
600
500
400
300
200
100
0
b
Fluorescence
(Rufoplaca)
600
500
400
300
200
100
0
c
Fluorescence
(phototrophic biofilm)
100
80
60
40
20
0
1E-5
1E-4
1E-3
0.01
0.1
1
Time (sec)


a
Rock surface temperature (°C)
Time (min)
b
Time (min)
X R P
30°C
40°C
50°C
60°C
70°C
80°C
Rock surface temperature

736