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Application of commercial biocides to lichens: Does a physiological recovery occur over time?

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ABSTRACT

Biocidal products are widely used to devitalize lichen thalli on monumental surfaces before their mechanical removal, but there is still lack of information about the persistence of the toxic effects over time. This issue is of paramount importance since it can greatly influence the process of lichen recolonization. The aim of this study was checking for physiological recovery or residual vitality of lichens after exposure to two commercial biocidal products, Biotin T or Preventol RI80. Samples of the foliose lichen *Xanthoria parietina* were treated with solutions containing the two biocides at the highest concentration suggested by the producer (3% and 2% respectively). Selected physiological parameters were investigated as indicators of sample vitality: photosynthetic parameters (F_V/F_M and PI_{ABS}), the content of chlorophyll *a*, chlorophyll *b*, beta-carotene, ergosterol and soluble proteins, after 24 and 72 hours and, to check for recovery, after 20 and 90 days. Both biocidal treatments induced severe physiological alterations, causing impairment to both the lichen photobiont and mycobiont, with Preventol showing a faster effect. The substantial loss of vitality following treatments with Biotin T and Preventol persisted over time, and no physiological recovery was found after 90 days.

1. Introduction

Lichens, symbiotic organisms composed by a heterotrophic (mycobiont) and an autotrophic (photobiont) partner, are involved in the biological colonization of external monuments and may be responsible for their biodeterioration (Caneva et al., 2008; Pinna, 2017). They colonize stonework whenever the conditions of moisture, light, temperature, and nutrition are favorable (Pinna, 2014). In particular, they are responsible for macroscopic alterations on monumental surfaces as a result of the release of several acidic substances and the penetration of hyphae into the superficial layers (Warscheid and Braams, 2000; Seaward, 2015). The relevance of their biological influence to the entire deterioration process and the interaction with non-biogenic agents should be evaluated very carefully (Salvadori and Municchia, 2016; Pinna, 2017). Besides accounting their aesthetic value/disvalue, there are some cases in which the removal of lichens is not advisable, e.g. a high biodiversity value of the communities and/or a bioprotective role (Pinna, 2014). However, when lichen removal is necessary, suitable techniques should prevent adverse side-effects to the stone substrate, to the operators, as well as to the surrounding environment (Caneva et al., 2008; Seaward, 2015). Among these techniques, the application of biocides is an effective method to devitalize lichen thalli before their mechanical removal (Rodrigues et al., 2011; Pinna, 2017) and, among biocides, quaternary ammonium compounds (QACs) and isothiazolinone compounds (OITs) are widely used biocidal active-compounds owing to their wide spectrum of action and their relatively low toxicity for humans and the environment (Richardson, 1988; Kumar and Kumar, 1999; Williams, 2007; Tezel and Pavlostathis, 2015).

The strong biocidal activity of QACs against fungi, algae and bacteria (Gilbert and Moore, 2005) depends on their mechanism of action, which includes perturbation of the cell wall and membrane structure, causing the release of cytoplasmic materials and the degradation of proteins and nucleic acids (Salton, 1968; Denyer and Stewart, 1998). OITs, being electrophile molecules, act on thiolic proteins, affecting protein synthesis, and the Krebs cycle, i.e. the ATP synthesis (Williams, 2007). The effectiveness of QACs and OIT-based biocides on lichens has been investigated in the field (e.g. De los Rios et al., 2009, 2012; Favero-Longo et al., 2017; Fonseca et al., 2010; Tretiach et al., 2007, 2010), however there is still a lack of information about the persistence of the toxic effects over time. This issue is of paramount importance

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since it can greatly influence the process of lichen recolonization. In addition, investigations about biocidal effects under controlled conditions are very scanty (Pinna, 2017). The aim of this study was thus to check for physiological recovery or residual vitality of lichens after exposure to two commercial biocidal products, under laboratory conditions.

2. Materials and methods

The response of the lichen green-algal photobiont has been assessed by means of the chlorophyll *a* fluorescence emission analysis, recognized as a tool for checking the vitality of photosynthetic organisms (Tretiach et al., 2008), as well as by the content of chlorophyll *a*, chlorophyll *b* and beta-carotene (Vannini et al., 2016). Ergosterol, the main sterol of the cell walls in fungi, has been used as indicator of the health state of the mycobiont (Vannini et al., 2016). The content of soluble proteins refers to both symbionts (Paoli et al., 2014).

2.1. Lichen material

Lichen communities that colonize stone substrates are often dominated by crustose species, which are hardly suitable for running lab experiments, in view of the difficulties in obtaining sufficient material for the analysis, and to have it reasonably clean from impurities like soil or mineral particles and crystals. Therefore, in this study, we used the foliose green-algal lichen *Xanthoria parietina* (L.) Th. Fr., which is routinely in use in our laboratory and has been already successfully used as a test-organism to evaluate the accumulation and toxicity of heavy metals (e.g., Paoli et al., 2014), herbicides (Vannini et al., 2015, 2016) and other contaminants (e.g., Vannini et al., 2017). Moreover, this species, albeit mostly epiphytic, commonly grows also on stone substrates, especially basic and eutrophicated ones. In addition, *X. parietina* can be regarded as a good model species, being representative of a large group of lichens frequently growing on monuments, with a relatively wide thallus rich in the orange secondary metabolite parietin, which often causes the main visible impact (Nimis et al., 1992). Branches colonized by *X. parietina* were collected in a rural area of Tuscany (Italy) far from direct pollution sources (43°14′07″ N, 11°20′26″ E). In the laboratory, samples were stored in a climatic chamber at 16 °C, 40 μmol m^{-2} s^{-1} photons PAR (photoperiod of 12 h) and RH = 65% until use.

2.2. Experimental design

Biocide solutions were prepared diluting the pure biocide in mineral water to the highest concentration suggested by the producer: 3% for the OIT-based Biotin T [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%); C.T.S. S.r.l., Altavilla Vicentina, Italy] and 2% for the QAC-based Preventol RI80 [Benzalkonium chloride (i.e. alkyl dimethyl benzyl ammonium chloride; approx. 80%) + isopropyl alcohol (2%) in water; Lanxess, Köln, Germany]. Lichen samples were soaked for 1 h either in Biotin T or Preventol solutions or water alone as control, roughly maintaining the ratio 10:1 between sample mass (mg) and relative volume of the solution (cm^3) as suggested by Vannini et al. (2015, 2016). After soaking, two batches dedicated to the evaluation of short-term physiological effects were transferred to the climatic chamber at 16 °C and 40 μmol m^{-2} s^{-1} photons PAR (photoperiod of 12 h) with RH = 85% and then used for the analysis after 24 and 72 h. Other two batches dedicated to the evaluation of long-term effects and possible recovery were tied on rigid plastic nets and exposed on the trunk of pine trees at the Botanical garden of the University of Siena. The area is a suitable habitat for lichen recovery: it has been already used for physiological experiments on lichens (e.g., Paoli et al., 2010), being relatively humid and with a low level of pollution (Loppi and Paoli, 2015). Samples were harvested both after 20 days from treatment, allowing a comparison with data collected during *in-situ* studies (Favero-Longo et al., 2017), and after 90 days, a time suitable to detect signals of recovery.

2.3. Chlorophyll a fluorescence

Twenty samples were randomly selected and processed as reported by Vannini et al. (2016, 2017). Samples were lightened with saturating red light for 1 s (650 nm, 3,000 μmol photons $m^{-2}s^{-1}$) with a light emitting diode (LED). Measurements were carried out through a Plant Efficiency Analyzer fluorimeter (Handy PEA, Hansatech, Norfolk, UK). Chlorophyll *a* fluorescence emission increases from F_0, when all the reaction centers of the PSII are open, to F_M, when all the reaction centers are closed. Results were expressed as F_V/F_M, an indicator of the maximum quantum yield of primary photochemistry (where $F_V = F_M - F_0$) and PI_{ABS}, a global indicator of photosynthetic performance (Strasser et al., 2004). The results of these tests were coupled with the analysis of the polyphasic transient of Chl_aF, plotted on a logarithmic scale (OJIP transient); this test is used for lichen photobionts as an integrative analysis for the evaluation of structural and functional information on the photosynthetic apparatus (Malaspina et al., 2014).

2.4. Ergosterol and photosynthetic pigments

Lichen samples (130 mg) were homogenized in 1 mL of absolute ethanol and then centrifuged at 22,000 rcf for 10 min at 4 °C. The resulting supernatant was filtered at 0.45 μm through a syringe filter and then stored at 4 °C. Samples were analysed by HPLC (Agilent 1100) using a C18 column (Phenomenex, 250 × 4.6 mm, particle size 5 μm) as separator. Ergosterol was separated using methanol as mobile phase at a flow rate of 1 mL/min. Runs were monitored at 280 nm. Chlorophyll *a*, chlorophyll *b* and beta-carotene were separated isocratically using methanol-acetone volumes (50-50) with a flow rate of 1 mL/min. Runs were monitored at 440 nm. Calibrations curves of chlorophyll *a*, chlorophyll *b*, beta-carotene and ergosterol were prepared by dissolving pure standards (Sigma-Aldrich, Germany) in absolute ethanol. Three replicates were used for each sample. The limits of quantification (LOQ) were: chlorophyll *a* = 2 μg/g, chlorophyll *b* = 3 μg/g, beta-carotene = 2 μg/g, ergosterol = 4 μg/g.

2.5. Soluble proteins

Lichen samples (about 50 mg) were homogenized with 1 mL of phosphate buffer solution (K_2HPO_4 50 mM and KH_2PO_4 50 mM, pH 6) and then centrifuged for 5 min at 22,000 rcf. An aliquot of 100 μL of the supernatant was added to 1.5 mL of the Bradford solution. After the reaction (about 15 min) samples were analysed by a spectrophotometer (Agilent 8453) at a wavelength of 595 nm. The calibration curve was prepared with albumin bovine serum diluted in the phosphate buffer solution at 0.125–1 mg/mL. Three replicates were used for each sample. LOQ was 1 μg/g.

2.6. Statistical analysis

Owing to the limited data-set, non-parametric statistics were used. A permutation test was used to check for differences ($p < 0.05$) between treatments and control samples, as well as between different treatments at the same time. Differences ($p < 0.05$) among the effect of one biocide at different times were checked with the Kruskal-Wallis ANOVA using the Dunn's test for post-hoc comparisons. For the statistical analysis, values below the LOQ were replaced by their respective LOQ value. To normalize the data and allow for meaningful comparisons, the effect of treatments was expressed as percentage ratios between treated to control samples. Results are presented as means ± bootstrapped 95% confidence intervals. All calculations were run using the free software R (R Core Team, 2017).