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

Manuscript Details

Manuscript number	IBB_2017_1147
Title	Application of commercial biocides to lichens: does a physiological recovery occur over time?
Article type	Full Length Article

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 (FV/FM and PIABS), 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.

Keywords
Biotin T;
ergosterol;
chlorophyll fluorescence;
Preventol;
soluble proteins;
toxicity

Taxonomy
Biocide

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- Biotin T and Preventol RI80 induced severe physiological alterations in the lichen *Xanthoria parietina*
- Preventol RI80 was more effective in causing faster damage
- No physiological recovery was detected after 90 days from treatment

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}), 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.

Keywords*:* Biotin T, ergosterol, chlorophyll fluorescence, Preventol, soluble proteins, toxicity

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 Casanova-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, 2004; Tezel and Pavlostathis, 2015).
The strong biocidal activity of QACs against fungi, algae and bacteria (Gilbert and Moore, 1999) 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, 2004). 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 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* Th. Fr. (L.), 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. 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 hours) 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 hours) with RH=85% and then used for analysis after 24 and 72 hours. 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 (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 chosen and processed as reported by Vannini et al. (2016; 2017). Samples were lightened with saturating red light for 1 sec (650 nm, 3000 µmol photons $s^{-1}m^{-2}$) 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 centres of the PSII are open, to F_M, when all the reaction centres 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). 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., 2004).

2.4. Ergosterol and photosynthetic pigments

Lichen samples (130 mg) were homogenized in 1 mL of absolute ethanol and then centrifuged at 22,000 rpm for 10 minutes 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 5µm) 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 50mM and KH_2PO_4 50Mm, pH 6) and then centrifuged for 5 minutes at 22,000 rpm. An aliquot of 100 µL of the supernatant was added to 1.5 mL of the Bradford solution. After the reaction (about 15 minutes) 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 mg/mL to 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. The Mann-Whitney U 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 interval. All calculations were run using the free software R (R Core Team, 2017).

3. Results

As expected, both biocidal treatments induced severe physiological alterations in the lichen *X. parietina* (Table 1). Compared with control values, Preventol RI80 altered all investigated parameters already after 24 h from treatment. Chlorophyll *a*, chlorophyll *b*, beta-carotene, ergosterol and soluble proteins showed time-dependent changes comparing early (24 and 72 h) with late effects (20 and 90 d), with lower values being detected after 20 and 90 days from treatment. Biotin T induced alterations mainly after 20 and 90 days from treatment. Early physiological effects (24 h) were detected in the photobiont, consisting in alteration of chlorophyll fluorescence emission (F_V/F_M and PI_{ABS}), and content of chlorophyll *b*. Ergosterol content was significantly reduced only after 72 hours. The content of chlorophyll *a*, beta-carotene and soluble proteins showed significant reductions only after 20 and 90 d from treatment.

Fluorescence transient curves (Figure 1) reflected a substantial loss of vitality in the photobiont following treatments with both Biotin T and Preventol, which persisted over time. Transient curves lost the typical sequence of inflection points (O-J-I-P steps) of healthy (control) samples. Fluorescence emission was characterized by an abrupt (24 h) reduction of F_M values, and a zeroing of both F_M and F_0 after 20 and 90 d. Although the ratio F_V/F_M in some cases significantly increased after 20 and 90 d from treatment, the flat profile of the curves and the reductions of chlorophyll *a*, chlorophyll *b* and beta-carotene content, indicated the absence of any physiological recovery.

4. Discussion

Both biocidal treatments caused overall severe physiological alterations to the lichen *X. parietina*, in the long time (20-90 d), but some temporal differences emerged in the short time (24-72 h), with Preventol being usually more effective.

Both biocides are characterized by the presence of a QAC as active ingredient: Preventol is composed only of the benzalkonium chloride (BZK) (about 80%) while the Biotin T, in addition to an isothiazolinone (7-10%), is composed of the dodecyl-dimethyl ammonium (40-60%). QACs are a class of membrane-active molecules effective on a broad range of microorganisms and commonly used as antiseptics and disinfectants (McDonnel and Russel, 1999). They are able to alter cell walls and membranes, leading to the leakage of intracellular materials and to the degradation of nucleic acids and proteins (Salton, 1968; Denyer and Stewart, 1998). In fact, the strong dissolution of intracellular solutes, as well as membrane constituents, caused in particular by benzalkonium chloride (BZK), can be the reason of a higher levels of beta-carotene, ergosterol and soluble proteins observed after 24 and 72 h from treatment with Preventol. The algicidal action of QACs has been well investigated under laboratory conditions: Walker and Evans (1978) reported that high concentrations of these compounds (between 3 to 5 ppm) inhibited the growth of *Spirodela oligorhiza* and caused the death of *Chlorella* sp.; other studies reported the occurrence of toxic effects on the physiology of *Chlorella pyrenoidosa*, *C. vulgaris* and *Scenedesmus quadricauda*

(Jing et al., 2012; Liang et al., 2013; Zhang et al., 2015; Zhu et al., 2010). Negative effects of QACs on chlorophylls and carotenoids have been reported also in higher plants, with damages being dose- and time-dependent (Biczak et al., 2017).

A faster action of Preventol on the photobiont of *X. parietina* can be explained by a greater algicidal action of BZKs compared with Biotin T compounds. BZKs alter the fluidity of cell membranes, leading to the dissolution of lipids contained in the phospholipid bilayer of cell membrane, which at once generates modifications of osmotic properties and affects physiological processes, such as cellular respiration, ion exchange and cell wall biosynthesis (e.g., Salt and Wiseman, 1970; Gilbert and Moore, 2005; Pérez et al., 2009). Also, n-octyl isothiazolinone (OIT) in Biotin T is reported as an efficient biocidal (Williams, 2004) with algicidal action, as shown in the alga *Selenastrum capricornutum* (EPA, 2007). OIT inhibits dehydrogenase enzymes involved in several steps of the Krebs cycle, blocking critical functions of the cellular metabolism, such as energy production and cellular respiration, and hence, cellular growth (Williams, 2007). Despite the algicidal action of OIT is proven, if we consider the relatively low persistence of this compound in the environment (EPA, 2007) and the lack of any information about its residence time in treated lichens, no assumption about its stability in these organisms can be made. Considering the proved stability of QACs (Li et al., 2014), we may argue that the loss of chlorophyll content in the lichen photobiont following Biotin T treatment could be mainly due to the action of dodecyl-dimethyl ammonium.

The apparent increase in F_V/F_M (about 11%) after 20 and 90 d does not indicate a physiological recovery, since the flat profile of fluorescence emission curves (OJIP transients), coupled with the reduction of F_0 over time, confirm the severe damage occurred to the photosynthetic apparatus of the photobiont. A similar loss of vitality (leading to flat OJIP transients) was observed during field treatments of crustose lichens (*Protoparmeliopsis murali*s and *Verrucaria nigrescens*) with a wide range of biocides, including Biotin T and Preventol (Favero-Longo et al., 2017).

Ergosterol is a fundamental component of the fungal membrane, being responsible for its functionality (Parks and Casey, 1995) and mechanical resistance to osmotic stresses (Dupont et al., 2012). The quantification of ergosterol is commonly used for the evaluation of fungal membrane integrity and functionality (Eklab et al., 1998; Sundberg et al., 1999), as well as for the assessment of the physiological status of the mycobiont in lichen thalli (Vannini et al., 2017). Ergosterol content reflected the damage endured by the lichen mycobiont: the loss of ergosterol after 72 h following Biotin T treatment was probably due to the interactive effect of QACs and OITs. Its complete degradation after 20 d irrespective of biocidal treatment is likely a consequence of the degradation of the fungal membrane caused by QACs; on the other hand, the lack of any recovery after 90 days could be related to the block of the biosynthetic pathway leading to its synthesis. In fact, ergosterol and cholesterol are the final products of the same biosynthetic pathway (Dupont et al., 2012), and cholesterol biosynthesis has been proven as sensitive to the effects of a particular class of QACs, namely BZKs (Herron et al., 2016). Accordingly, alterations of the biosynthetic pathway are plausible also in the case of ergosterol following exposure to QACs.

Reductions in the content of soluble proteins were observed for Biotin T (71%) and Preventol application (41%) after 20 and 90 days. QACs are known to alter the functionality of proteins, especially those linked to the phospholipid bilayer (Gilbert and Moore, 2005). Furthermore, OIT compounds may cause a damage to thiol-proteins, both soluble and insoluble, as shown in *Saccharomyces cerevisiae*, where protein degradation was considered the main cause of cellular death (Williams, 2007). Therefore, a higher damage to soluble proteins after 90 days following Biotin T exposure is likely due to the synergic effects of OITs and the QACs.

Finally, after 90 days our results suggest the occurrence of irreversible damages in *X. parietina* after both biocidal applications, so that these biocides can be considered interchangeable for lichen

removal during stone treatments. Since phenomena of resistance or residual vitality were detected in crustose lichens (*V. nigrescens*) even 180 days after the treatments (Favero-Longo et al., 2017), a recovery after longer periods cannot be completely excluded. In fact, 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 specific thresholds (F_V/F_M<0.150 and F_0 decreased by 80%) (Favero-Longo et al., 2017), so that the question of a species-specific resistance to biocidal compounds remains open. With this regard, *X. parietina* exhibited a remarkable sensitivity to glyphosate (Vannini et al., 2016), but the same herbicide was instead quite ineffective against other lichen species i.e. *V. nigrescens* and *P. muralis* (Favero-Longo et al., 2017).

On the other hand, even after an effective devitalization of the thalli, the dispersion of non-dead propagules during the mechanical cleaning (Seaward, 2004) and/or the arrival of new propagules from the surrounding environment (Nascimbene et al., 2009; Favero-Longo et al., 2014; 2017) enhance lichen recolonization after restoration works (Nascimbene et al., 2008; 2009), which is, nevertheless, an unavoidable process. In this context, it is worth noting that both research and field evidence discourage the widespread use of nitrogen-containing biocides like QACs, which can act as nutrients for micro- and macro-organisms, and thus favour recolonization phenomena (Pinna, 2017). Moreover, QAC resistance and QAC-induced antibiotic resistance are increasingly reported due to the wide QAC use in disinfectant formulations, and their consequent release and persistence in the environment, suggesting that QACs should be generally cautiously used to reduce/avoid adverse impacts to both humans and the environment (Tezel and Pavlostathis, 2015). Thus, the development of alternative control methods is of primary importance (Pinna, 2017).

5. Conclusions

Treatments with both Biotin T and Preventol induced severe physiological alterations in the lichen *X. parietina*. Our results showed great changes in chlorophyll *a* fluorescence emission, as well as the content of photosynthetic pigments, ergosterol and soluble proteins already after 24 h from treatment, with Preventol being more effective to generate rapid physiological alterations. After 20 days from the application, both biocides produced physiological impairments to the photobiont and the mycobiont. The substantial loss of vitality following the treatments with Biotin T and Preventol persisted over time. After 90 days, no physiological recovery was observed.

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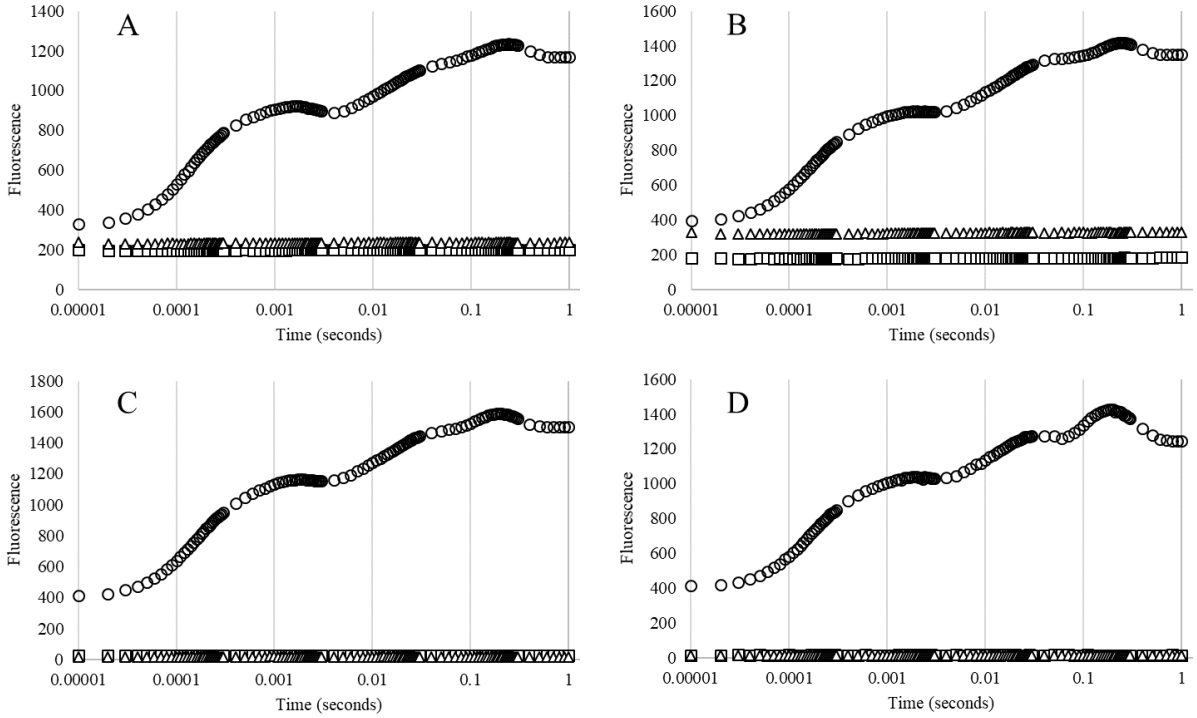

Fig. 1. OJIP fluorescence transients of lichen samples treated with Biotin T (□), Preventol (Δ) and mineral water (○) (used as control), after 24 (A) and 72 hours (B) and 20 (C) and 90 days (D) from the applications. Each transient represents the average of five replicates.

Table 1. Physiological parameters (% of control) in samples of the lichen *Xanthoria parietina* after biocidal treatments (means ± 95% confidence interval). Different letters indicate statistically significant differences over time; values in bold indicate significant differences between treatment and its respective control; italics indicates significant differences between treatments at the same time.

Time	Chlorophyll *a*		Chlorophyll *b*		Beta-carotene		Ergosterol		Soluble proteins		F_V/F_M		PI_{ABS}	
	Bio	Prev	Bio	Prev	Bio	Prev	Bio	Prev	Bio	Prev	Bio	Prev	Bio	Prev
24 h	*139±59a*	***7±3a***	***243±96a***	***0.5±0.1a***	*132±68a*	***209±105a***	*90±29a*	***157±40a***	*101±17a*	***225±35a***	**4±2a**	**4±1a**	**0.1±0.1a**	**0.1±0.1a**
72 h	*121±27a*	***7±2a***	***7±2b***	***0.5±0.1a***	*86±18a*	***230±62a***	***54±9a***	***162±42a***	*127±10a*	***228±48a***	**8±5ab**	**3±1a**	**1±1a**	**0.1±0.1a**
20 d	**3±3b**	**0.1±0.01b**	**2±2c**	**0.3±0.04b**	**4±4b**	**3±3b**	**4±1b**	**2±2b**	***27±2b***	***66±5b***	**11±4b**	**17±6b**	**0.4±0.4a**	**2±2a**
90 d	**0.1±0.01b**	**0.1±0.01b**	**0.3±0.04c**	**0.3±0.03b**	**3±0.2b**	**3±0.2b**	**0.4±0.05c**	**0.4±0.03b**	**30±16b**	**52±23b**	**15±6b**	**20±7b**	**0.1±0.01a**	**0.1±0.01a**

The Authors declare no conflict of interest