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Biotechnological applications of lichen phycobionts: fast bioassay of environmental toxicity

Rosa de las Heras[1] · Myriam Catalá[1]

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Abstract

Microbioassays allow for efficient contamination monitoring and control strategies. Free-living microalgae, representative of the aquatic environment, are the most used organisms due to high sensitivity and reproducibility. However, a lack of testing methods representative of terrestrial habitats has long been highlighted. A good unexploited option is the use of lichen phycobionts. The use of appropriate biomarkers leads to a reduction in costs and number of organisms, contributing to cost-efficient, rapid, and sensitive microbioassays. With the aim to develop a fast microbioassay, axenic *Asterochloris erici* was grown on treated cellulose paper, desiccated and rehydrated with different concentrations of inorganic and organic pollutants. Chlorophyll autofluorescence and free radical content were measured 5 min post-rehydration as energetics and oxidative status biomarkers respectively. Fluorescence microscopical images of exposed phycobionts were also collected. Potassium dichromate and copper sulphate decreased chlorophyll autofluorescence at high concentrations whereas boric and clofibric acids had little effect, all showing LOECs similar to those found in the literature. Heavy metals induced free radical bursts at extremely low concentrations whereas boric and clofibric acid showed modest and fluctuant increases. Microscopical images support fluorometric results and relate free radical bursts with bigger cells. In every case, free radicals LOEC is lower than chlorophyll autofluorescence's by at least three orders of magnitude, making this microbioassay highly sensitive and fast, as well as low cost and ecologically relevant.

Keywords Microbioassay · *Asterochloris erici* · Pollutants · Chlorophyll autofluorescence · Free radicals · Microalgae

1 Introduction

The adopted legislative measures have partly contributed to mitigate the risks arising from chemical pollution of water. However, the growing demand and the discovery of new pollutants require the continuous protection of human health and the environment through research (Damià and López 2008). There are compounds that are only partially removed from wastewater treatment plants and are persistent in surface waters (Winkler et al. 2001). For example, boron exists naturally in the form of borates but has increased considerably in the aquatic environment through human activities (Butterwick et al. 1989). Boron is one of the most important contaminants in most coal ash materials (Adriano 2001). It is estimated that 11,800 t are released annually into coal fly ash from coal combustion (Bertine and Goldberg 1971). This is an essential micronutrient for plant growth and its deficiency affects the functioning of several physiological and metabolic processes (Lukaszewski and Blevins 1996). Boron toxicity generally occurs in semi-arid and arid environments, where the level of B is high in the soil or in irrigation water (Nable et al. 1997). High levels of boron are found in domestic wastewater (0.5 to 2 mg/L) in these regions (Polat et al. 2004) while levels in raw water have been reported in the low μg/L range (Health Canada 2020). B is only available to plants in water-soluble forms such as boric acid or borate anions (Shorrocks 1997). Boron has several mechanisms of toxicity in plants, it can disturb the development of the cell wall or the alteration can be metabolic. It can also affect cell development and division and if there are high concentrations in the leaves, an osmotic imbalance can occur (Reid et al. 2004). Increases in reactive oxygen species (ROS) induced by B have been reported in tobacco, tomato and chickpea crops in concentrations range 0.05–64 mM (García et al. 2001; Ardic et al. 2009; Cervilla et al. 2009).

✉ Myriam Catalá
myriam.catala@urjc.es

Rosa de las Heras
mrdelasherasglez@gmail.com

[1] Department of Biology, Geology, Physics and Inorganic Chemistry (Area of Biodiversity and Conservation), ESCET, Rey Juan Carlos University, 28933 Madrid, Móstoles, Spain

Organic pollutants, as clofibric acid, can bioaccumulate and cause adverse effects in the environment (Filimonova et al. 2016). Its environmental persistence is estimated at more than 21 years (Emblidge and DeLorenzo 2006) and water residence time is 1–2 years (Zuccato et al. 2000). Concentrations as high as 7300 ng/L have been reported in groundwater samples (Heberer et al. 1995). In river waters concentrations higher than 0.001 mg/L have been found (Stumpf et al. 1999). It is the active metabolite of clofibrate, one of the first compounds that was used as a lipid regulator (Kümmerer 2008). Additionally, (R) -mecoprop is a structural isomer of clofibric acid used as a herbicide in cereal crops (Buser et al. 1998). Exposure to high concentrations (0.5, 1.0 and 2.0 mg/L) of clofibric acid increased the activity of antioxidant enzymes in *Typha* species and induced oxidative damage (Dordio et al. 2009).

Heavy metals are within the most frequent non-biodegradable pollutants reported to be highly concentrated (Mallick and Rai 2002). Besides the fact that chromium appears naturally, it is also released into the environment by industrial processes (Bluskov et al. 2005). Hexavalent chromium is harmful to wildlife for concentrations higher than 0.05 ppm (Khalil et al. 1998) and carcinogenic to humans (Caravelli et al. 2008). Environmental levels in freshwater range 0–117 μg/L (mean 9.7 μg/L) (Shanker and Venkateswarlu 2011), however levels above 12 mg/L and 550–1500 ppm/L of chromium have been observed in groundwater (Jaishankar et al. 2014). Progressive stages of chlorosis and necrosis have long been detected due to the toxicity of Cr in plants (Hauschild 1993). In crops, when Cr is available in concentrations of 1–5 mg/L, alterations are produced in the metabolic processes (Dube et al. 2003). Corradi and Gorbi (1993) demonstrated that Cr (IV) supplied as potassium dichromate inhibited cell proliferation and coenobium formation in *Scenedesmus acutus*. Copper is an element of widespread use in industry and agriculture (Fernandes and Henriques 1991). Quantities of 1.3–1.6 kg/ha per year of pesticides containing copper sulphate have been documented, frequently used to remove phytoplankton blooms in municipal water supplies (Haughey et al. 2000; He et al. 2005). Copper levels in runoff water can fluctuate between 5 and 200 μg/L (Wu et al. 1996; Sansalone and Buchberger 1997) and WHO has considered environmental concentrations in surface waters to range in the low μg/L range (WHO 2004). Despite, spinach shows a high tolerance to Cu, if treated at high concentrations (around 10^3 mg/L), it suffers increases in lipid peroxidation products and activities of antioxidant enzymes and decreases in chlorophyll content to some degree (Gong et al. 2019). The effects of copper sulphate on photosynthesis-related gene transcription on cyanobacteria *Microcystis aeruginosa* were demonstrated (Qian et al. 2010).

The lack of testing organisms representative of terrestrial habitats in microbioassays has long been highlighted (Catala et al. 2009). A good unexploited and unknown option is the use of lichen microalgae (Dominguez-Morueco et al. 2014). These have tolerance to desiccation until rehydration allows them to reset their metabolism (Bewley 1979), and are capable of growing outside of symbiosis (Wolseley and Hawksworth 2009). Many species of lichens are associated with the alga *Asterochloris erici* (formerly *Trebouxia erici*) (Skaloud and Peksa 2010) which is sensitive to micropollutants (Dominguez-Morueco et al. 2014), may be obtained in large quantities at low cost and tolerates a wide temperature range. The use of biomarkers in phycobionts may help detect the presence of pollutants in a short period of time and at low cost. The most widely used biomarker to estimate the photosynthetic activity is chlorophyll autofluorescence. The integrity of the plant chlorophyll may be altered and affect photosynthesis. The yield of chlorophyll autofluorescence depends on the conditions in which the organism is found, if exposed to contaminants or suboptimal temperatures, the fluorescence emission will be lower (Catala et al. 2010). Rehydration of lichen thalli and axenic phycobionts was shown to cause an intracellular burst of free radicals (Catala et al. 2010) derived from reactive oxygen species (ROS) whose intensity is modified in the presence of environmental pollutants (Álvarez et al. 2015).

To comply with current environmental assessment necessities, new microbioassays developed should be cost-efficient, rapid and sensitive. Rapid biomarkers might contribute to this intention. The use of an aero-terrestrial microalga as *Asterochloris erici* could also contribute to microbioassays presenting a great biological and ecological relevance. Therefore, the following objectives are pursued: (1) designing a cost-effective and rapid microbioassay for the detection of pollutants in environmental samples based on the lichen phycobiont *Asterochloris erici*, (2) finding a suitable rapid biomarker of toxicity in this species.

2 Material and methods

2.1 Biological material

An axenic strain of the lichen alga *Asterochloris erici* (formally *Trebouxia erici*, SAG 32.85 = UTEX 911; collection of algae of the University of Texas at Austin, TX, USA) described by Skaloud and Peksa (2010) was used. This phycobiont formerly known as *Trebouxia erici* (Ahmadjian 1993), was isolated from the lichen *Cladonia cristatella* Tück. The strain was kindly provided by Dr. Gasulla and Dr. Barreno (University of Valencia, BITI, Valencia, Spain).

2.2 Chemicals

Magnesium sulphate heptahydrate, sodium chloride, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, zinc sulphate heptahydrate, manganese (II) chloride tetrahydrate, molybdenum oxide (VI), copper (II) sulphate pentahydrate, cobaltous nitrate hexahydrate, potassium hydroxide-ethylene diamine tetra—acetic acid solution, ethylenediaminetetraacetic acid disodium salt dihydrate, potassium hydroxide, ferrous sulphate heptahydrate and sulfuric acid for 3 N Bold medium were supplied by Panreac (Barcelona, Spain) with a minimum of purity of 98% except molybdenum oxide (VI) and potassium hydroxide with a purity of 85%. Sodium nitrate and glucose were obtained from Sigma-Aldrich (Steinheim, Germany) with a minimum purity of 99% and casein was obtained from Fluka Biochemika (San Sebastian Spain) with impurities less than or equal to 3.5% chloride (expressed as sodium chloride). European agar was acquired from Conda-Pronadisa (Valladolid, Spain).

The phycobionts were exposed to five different concentrations of the following pollutants in a logarithmic range: potassium dichromate ($K_2Cr_2O_7$, CAS #777850–9) (10^{-6} to 10^5 mg/L), copper sulphate (Cu_2SO_4, CAS #7758-98-7) (4×10^{-9} to 4×10^5 mg/L), boric acid (H_3BO_3, CAS #10043–35-3) (10^{-5} to 10^4 mg/L) and clofibric acid ($C_{10}H_{11}ClO_3$, CAS #882–09-7) (5×10^{-5} to 5×10^3 mg/L). Potassium dichromate, copper sulphate and boric acid had a purity of at least 98% and were acquired through Panreac (Barcelona, Spain). Clofibric acid had a purity of 97% and was acquired through Sigma-Aldrich (Steinheim, Germany).

2.3 Phycobiont culture

Asterochloris erici was grown on 0.6 cm diameter discs of pure cellulose treated with sulfuric acid which improves its non-stick properties and make it waterproof, commercially available as oven paper (Lanta alimentary trade mark, distributed by ATEMPO FOODPACK SA, Barcelona, Spain). Discs were previously washed in deionized water, autoclaved and oven-dried at 60 °C, laid on semisolid 3 N Bold medium (Harold and Parker 1962) containing 10 g casein hydrolysate, 20 g glucose and 15 g agar per liter (Ahmadjian 1960). An amount of $2{,}5 \times 10^6$ cells per disc were seeded and the cultures were maintained in a growth chamber at 19 °C under a 12:12 dark/light cycle (lighting conditions 25 μmol m^{-2} s^{-1}).

2.4 Desiccation and rehydration treatments

The discs were removed from the culture medium after 21 days and were placed in a closed container with silica gel for desiccation in the growth chamber. The microalgae were totally desiccated in less than 60 min. If the rehydration was to be done the same day, desiccated phycobionts were kept at room temperature. If this process was to be done the next day, the desiccated phycobionts were maintained at 4 °C in the same container.

For rehydration with the different treatments, the discs with the desiccated phycobionts were deposited in black flat bottom 96-multiwell plates and rehydrated with 100 μL of the toxicant solutions. To obtain a final concentration of 10 μM, 10 μL 2,7- dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) were added to every sample to analyze free radical production (Álvarez et al. 2015). Five replicates of each concentration were performed. The experimental control was deionized water. After 5 min of rehydration, the chlorophyll autofluorescence and the production of intracellular free radicals were measured.

2.5 Chlorophyll autofluorescence and intracellular free radical production measurement

The chlorophyll autofluorescence and intracellular free radical production as dichlorofluorescein (DCF) fluorescence were measured in a Synergy™ HTX Multi-Mode Microplate Reader (Izasa Scientific Biotek) with an excitation filter of 485 nm. Emission of chlorophyll autofluorescence was analyzed at λ_{em} 635 nm and DCF fluorescence was measured at λ_{em} 528 nm (Álvarez et al. 2015). Preliminary assays demonstrated that fluorescence increased abruptly for both parameters in the first seconds to attain a steady state from 1 to 15 min. After analysing these biomarkers, the rehydrated phycobionts were cut and observed under the fluorescence microscope. The fluorescence images obtained were treated with ImageJ processing software.

2.6 Statistics

Data are expressed as the mean ± the standard error of the mean. Five replicates for each treatment were used. The Student t-test was used to determine if there are statistically significant differences between treatments and controls using *Rterm* (R-UCA packet). In any case it was considered as significant a p value <0.05. The Lowest Observed Effect Concentration (LOEC) is the lowest concentration for which the observed response is significantly different respect to control and the No Observed Effect Concentration (NOEC) is highest concentration in which the response doesn't present statistically significant differences.

3 Results

The phycobionts were rehydrated with potassium dichromate, copper sulphate, boric acid or clofibric acid and fluorescence

was recorded after 5 min. LOEC and NOEC were noted for each biomarker.

When analysing the effects of potassium dichromate on chlorophyll autofluorescence, we observe a slight increase at 1 mg/L indicative of hormesis, where the LOEC is set (Fig. 1a). It decreases consistently in the range of 10^2–10^5 mg/L, falling well below controls (<40%) indicating a strong toxicity. Upon rehydration with copper sulphate, values decrease under 80% at 4×10^3 mg/L where LOEC is registered (Fig. 1b), no hormetic effect is observed. In the case of boric acid, values fluctuate slightly below controls, the NOEC and LOEC are recorded at 10^{-1} mg/L and 1 mg/L respectively (Fig. 1c). For clofibric acid, chlorophyll autofluorescence increases above 120% from 5 x 1 mg/L. The LOEC is recorded at 5 x 10 mg/L (Fig. 1d).

In the rehydrated microalgae, the production of free radicals bursts from 10^{-4} mg/L of potassium dichromate (LOEC), it remains stable in the range of 10^{-2} to 10 mg/L and then increases abruptly again to reach its maximum at the concentration of 10^3 mg/L, then dropping sharply at 10^5 probably indicating a metabolic failure (Fig. 2a). With copper sulphate, a significant increase in the values of free radicals above 200% is observed for very low concentrations of 4×10^{-7} mg/L (LOEC) (Fig. 2b). From this concentration on, the values remain constant up to 4×10^2 mg/L where a peak above 700% is observed, to decrease very sharply below controls for the successive higher concentrations also likely as a result of a metabolic failure. Regarding boric acid, slight increases in the values of free radicals ranging from 120% to 140%, are observed all along the concentration interval. LOEC and NOEC values are recorded at 10^{-4} mg/L and 10^{-5} mg/L, respectively (Fig. 2c). In the rehydration with clofibric acid, the highest value of free radicals occurs at concentrations of 5×10^2 mg/L. At the next concentration, the value decreases sharply. However, LOEC is already recorded at 5 x 10^{-4} mg/L at values above 120% (Fig. 2d). LOEC values have been collected in Table 1 showing that free radicals systematically report toxicant effects for concentrations at least 3 orders of magnitude lower than chlorophyll autofluorescence which seems much less sensitive.

Using florescence microscopy, we observed chlorophyll and DCF fluorescence in the algal cells 2–3 h post rehydration. The control corresponds to rehydration with deionized water (Fig.3A, A_1 and A_2). Chlorophyll autofluorescence of phycobionts rehydrated with potassium dichromate and copper sulphate (Fig. 3B and C respectively), do not differ visibly from the control (Fig. $3B_1$ and $3C_1$) but the fluorescence of free radicals is increased for both (Fig. $3B_2$ and $3C_2$). Whereas the increase caused by dichromate is more or less homogeneous, in the case of sulphate some phycobionts show clearly more fluorescence than others. The size of *Asterochloris erici* cells is not homogeneous and green fluorescence seems augmented in bigger cells. On the other hand, the fluorescence of chlorophyll is clearly higher than in controls in the case of boric and clofibric acids (Fig $3D_1$ and $3E_1$). The fluorescence of free radicals also increases heterogeneously in some cells with respect to the control (Fig. $3D_2$ and $3E_2$) but the association with bigger cells is not so evident.

4 Discussion

The main environmental regulatory agencies recommend analyzing the viability of algal cultures to determine the toxic

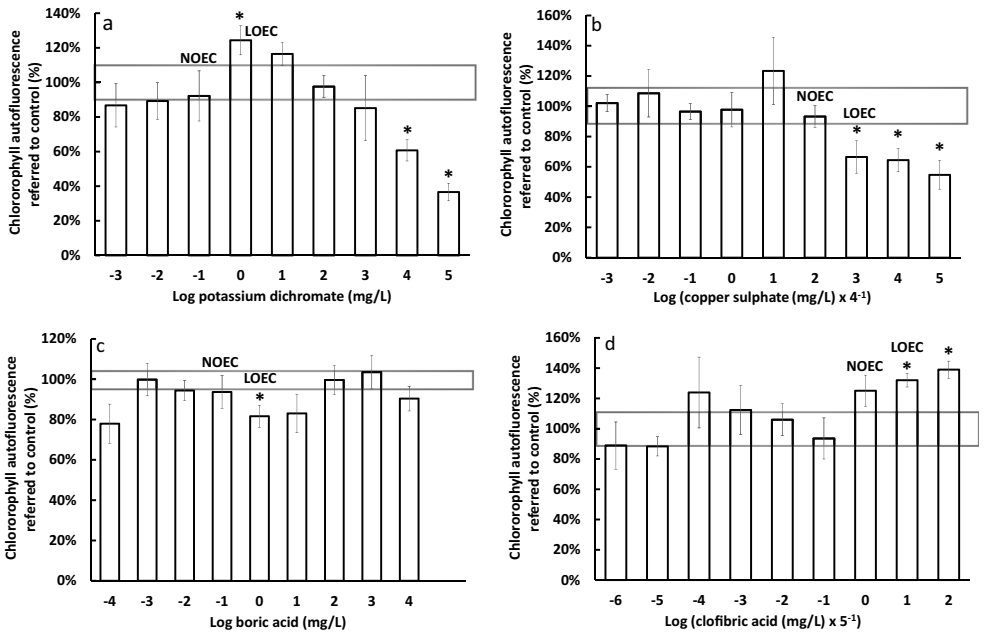

Fig. 1 Rehydrated *Asterochloris erici* chlorophyll autofluorescence referred to deionized water controls 5 min post rehydration with different concentrations of: a) potassium dichromate; b) copper sulphate; c) boric acid; d) clofibric acid. The error bars show the standard error of the mean. The solid lane frame stands for standard error of controls

Table 1 Values of Lowest Observed Effect Concentration (LOEC) according to each treatment applied and according to each biomarker analysed

	Potassium dichromate	Copper sulphate	Boric acid	Clofibric acid
LOEC VALUES (mg/L)				
Chlorophyll auto-fluorescence	1	4×10^{3}	1	5×10
Intracellular free radicals	10^{-4}	4×10^{-7}	10^{-4}	5×10^{-4}

effect of pollutants. Despite aquatic microalgae have been frequently used, due to high reproducibility and sensitivity (Shitanda et al. 2009), lichen phycobionts constitute an unexploited option which is highly representative of terrestrial ecosystems (Dominguez-Morueco et al. 2014; Traba et al. 2017). Their use would imply lower costs compared to plant testing and therefore greater efficiency in the control and monitoring of contamination. Among other features, they are available throughout the year, allow easy cultivation in the laboratory and tolerate dehydration (Casano et al. 2011). Furthermore, the ability of phycobionts to grow on solid surfaces such as nylon (Álvarez et al. 2015) or nitrocellulose (Gasulla et al. 2009), as they would in their natural environment, presents several advantages. These solid surfaces can be handled (including freezing and desiccation), stored, and transported easily, for example for adaptation to portable or field environmental testing kits. In this work we have particularly proven the advantage of using sulphuric acid treated paper, commercially available as oven paper. This support joins the advantages of formerly used materials such as nylon or nitrocellulose with low cost.

The measurement of sublethal biomarkers upon rehydration of axenic phycobionts cultured has allowed us to obtain efficiently massive and very rapid toxicity data. We have not found any literature referring methods that address toxicity during rehydration in other organisms adapted to anhydrobiosis so this is an innovative approach that takes into account relevant biological traits of some eukaryotic organisms likely involved in ecotoxicology and biodiversity loss. Chlorophyll autofluorescence provides us with a biomarker related with chlorophyll integrity and photosynthetic cell energetics (Krause and Weis 1984). Intracellular free radical release relates with oxidative stress and the cell metabolic status. Our results show that substances of different nature render diverse patterns of cellular response dependent on the biomarker chosen. It is noteworthy that LOEC values obtained from the toxicant effect on free radical release occurs at concentrations at least three orders of magnitude lower than effects on chlorophyll autofluorescence demonstrating the sensibility of this parameter.

Data of the toxicity of the chosen pollutants on microalgae are scarce despite their relevance in the environment. The LOEC obtained for potassium dichromate with chlorophyll autofluorescence is in the same order of magnitude to that found in the literature for growth inhibition tests of *Chlorella* (Friis et al. 1998) or *Selenastrurn capricornutum* (now *Raphidocelis subcapitata*) (Nyholm et al. 1992). We must highlight that the LOEC is set for a hormetic increase in chlorophyll autofluorescence probably due to compensatory mechanisms. Decreases indicating severe toxicity are

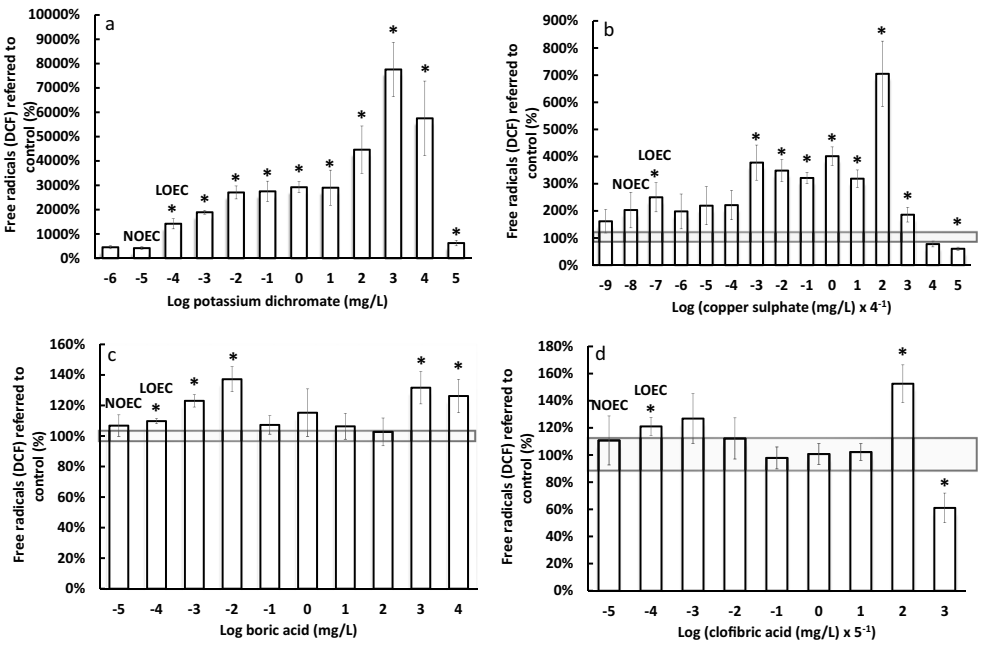

Fig. 2 Rehydrated *Asterochloris erici* free radical levels as DFC fluorescence referred to deionized water controls 5 min post rehydration with different concentrations of: a) potassium dichromate; b) copper sulphate; c) boric acid; d) clofibric acid. The error bars show the standard error of the mean. The solid lane frames the standard error interval of controls

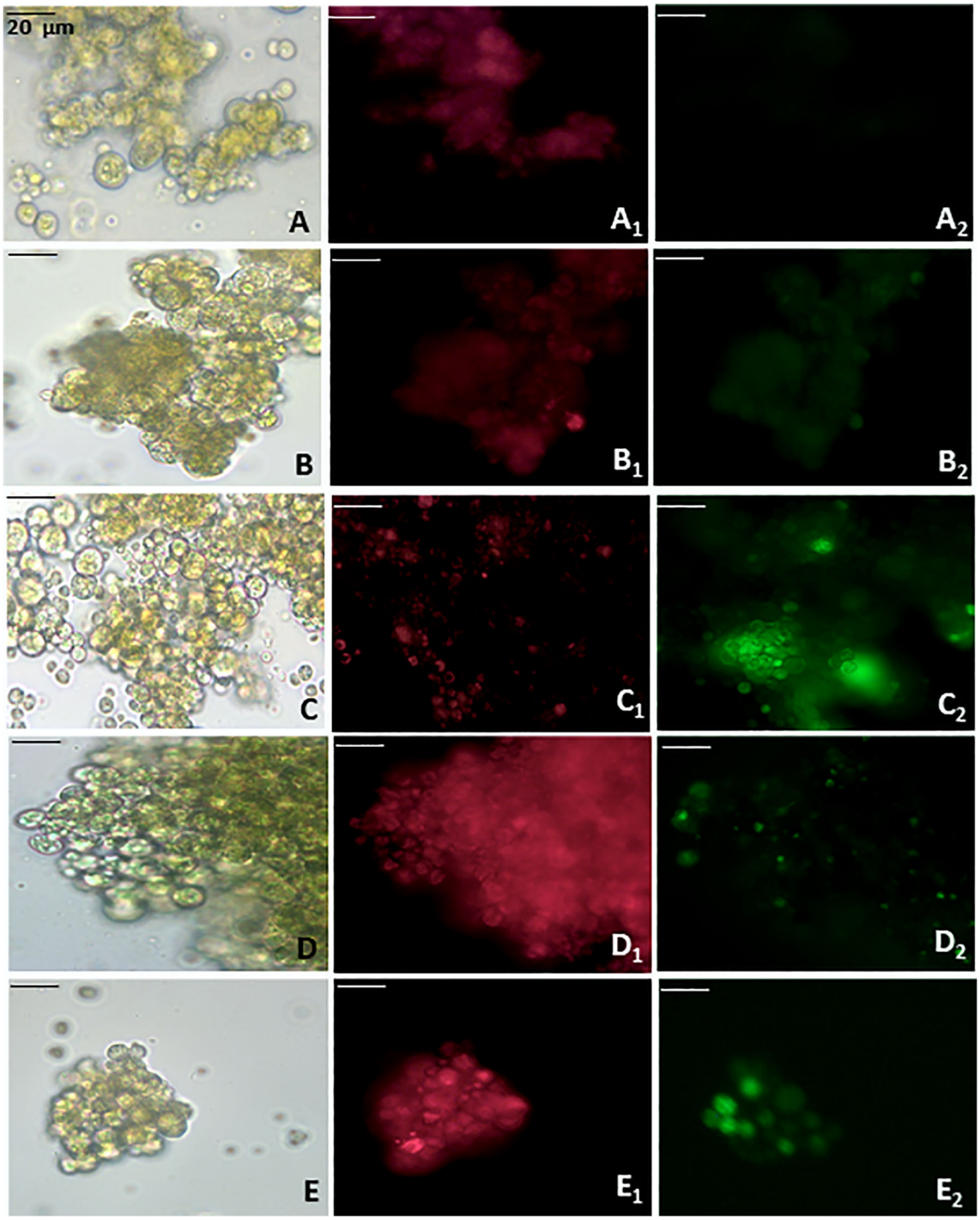

Fig. 3 In vivo fluorescence imaging of the cells of *Asterochloris erici* after rehydration with different treatments (LOEC values) using the fluorescent probe $DCFH_2$-DA. Red channel, chlorophyll autofluorescence (subscript 1). Green channel, DCF Fluorescence (subscript 2). Capital letters without subscripts are bright field images; A) Control, B) Potassium dichromate, C) Copper sulphate, D) Boric acid and E) Clofibric acid

observed at higher concentrations (10^4 mg/L). Despite copper sulphate has long been characterized as very toxic to algae, and is commercially used as an effective algicide, the effects on *A. erici* chlorophyll autofluorescence exhibit extremely high LOEC values (4×10^3 mg/L) very far from the µg/L range reported for *Chlorella vulgaris* (Greenfield 1942). In growth inhibition experiments, performed with *Selenastrum capricornuturn*, much lower EC50 values of 0.0467 mg/L were observed (Nyholm et al. 1992). The phycobiont used in our microbioassay may have some tolerance to the effects of copper. Photobionts have mechanisms for detoxification of heavy metals similar to other microalgae (Bačkor and Fahselt

2008). The effects of prolonged exposure to copper of the photobiont *Asterochloris erici* and the free green algae *Scenedesmus quadricauda* were compared and the photosynthetic apparatus of *Asterochloris erici* was protected more effectively against oxidative stress than in the case of *Scenedesmus quadricauda* (Piovár et al. 2011). The stability of *A. erici* chlorophyll status to transition metal species might be related to the high tolerance of lichens in general to minerals, a necessary evolutive adaptation due to their lack of cuticles or specific protection structures (reviewed in Expósito et al. 2020).

After the rehydration of the phycobionts with boric acid, the chlorophyll autofluorescence does not seem to be affected. A similar tolerance to boron has been also observed for *Chlorella vulgaris* (Chen and Pei 2016). Despite some authors reported that excess boron in plants deteriorates photosynthesis (Sotiropoulos et al. 2002) others found no significant effects (Reid et al. 2004). Clofibric acid does not lead to important decreases in chlorophyll autofluorescence either, in accordance with former studies in plants and aquatic microalgae. Other studies have shown that mecoprop herbicide (structural isomer of clofibric acid) does not exhibit high toxicity on non-target plant species. Kirby and Sheahan (1994) tested the toxicity of the herbicides isoproturon, atrazine and mecoprop on the green algae *Scenedesmus subspicatus*. The herbicides isoproturon and atrazine showed up to 3 orders of magnitude more toxicity than mecoprop. Chronic exposure of *P. subcapitata* rendered LOEC values of 150 mg/L for growth inhibition (Ferrari et al. 2003). Here we report a LOEC of 50 mg/L in a very short acute exposure of 5 min which would point to a much higher sensitiveness of the phycobiont *A. erici* to this pharmaceutical compared to aquatic microalgae, reinforcing the idea that diverse organisms must be used in testing methods for higher ecological relevance of results.

When we analyze the suitability of intracellular free radical levels as toxicity biomarker, our microbioassay shows an extraordinary sensitiveness rendering extremely low LOECs, especially compared to chlorophyll autofluorescence. Again, the lack of toxicity data of the pollutants on microalgae limits the discussion of our results. Potassium dichromate produces an intense burst of free radicals to *Asterochloris erici* at very low concentrations, LOEC is found at 10^{-4} mg/L, until the cell diminishes production likely due to serious toxic effects and metabolic failure, coinciding with chlorophyll autofluorescence decline. Hexavalent chromium has been reported to cause a marked oxidative stress finally leading to DNA damage and genotoxicity (Labra et al. 2007). Copper sulphate also induces a rapid and marked increase in free radicals from concentrations as low as 4×10^{-7} mg/L. It is not as intense as in the case of chromium, however, the decrease under control levels indicating a possible metabolic failure, is met at 10^{3}–10^{4} mg/L, also coinciding with chlorophyll autofluorescence decline.

Boric acid induces non dose-dependent slight increases in free radical content that achieve statistical significance at 10^{-4} mg/L. This is in accordance with the increases of lipid peroxidation observed for acute (24 h) exposure of *Chlorella vulgaris* (Chen et al. 2019). Similarly, intracellular free radical content is slightly increased at some concentrations of clofibric acid. In this case, after reaching a maximum at 10^{2} mg/L, levels decrease under controls probably indicating a metabolic failure. Interestingly, in this range of concentrations chlorophyll autofluorescence is increased. In the macrophyte *Typha* spp. (cattail) high concentrations of clofibric acid do not seem to affect the photosynthetic pigments but alters antioxidant enzymes activities (Dordio et al. 2009). Zhang et al. (2019) reported differences in cell size of *Chlorella pyrenoidosa* cultures treated with clofibric acid and other human pharmaceuticals. This could be related with the differences in the content of free radicals of the bigger cells observed by fluorescence microscopy, indicating that the sensitiveness of the microalgae may depend upon the physiological state of the culture.

This microbioassay aims to detect the toxicity of a sample in a very rapid, cost-efficient and sensitive way. Other bioassays performed with microalgae have other toxicity endpoints such as growth inhibition or biomass, and therefore require longer times (a minimum of 24 h). We did not get the expected answer by analysing the biomarker of chlorophyll autofluorescence. However, we obtain a very sensitive response analysing the intracellular free radicals although there is not much published literature on microalgae to compare our results. For example, while reported dichromate LOEC for *Chlorella* bioassays are in the high μg/L (820 μg/L) (Friis et al. 1998), we obtain LOEC values in the high ng/L range (10^{-1} μg/L) turning our bioassay into a much more sensitive tool able to detect Cr in the low μg/L range reported for freshwater (mean ca. 10 μg/L) (Shanker and Venkateswarlu 2011). Regarding copper sulphate, the LOEC values (4×10^{-1} ng/L) are extremely low but difficult to compare with bioassays using *S. capricornuturn* (EC_{50} 46.7 μg/L) (Nyholm et al. 1992). In any case, it perfectly suits the needs for the detection of surface waters reported levels, in the low μg/L range, comparingly reducing time and costs (WHO 2004). Boron induces non-dose-dependent free radical increases, a result similar to those obtained after analysis of acute 24 h exposure in *C.vulgaris* (Chen and Pei 2016). The LOEC value (10^{-1} μg/L) obtained is very low in relation to the range found in domestic wastewater (0.5 to 2 mg/L) (Polat et al. 2004) and even in environmental raw water assessment (low μg/L) (Health Canada 2020). In the same way, with clofibric acid, very low values of LOEC are obtained (5×10^{-4} mg/L) compared to those reported in

P. subcapitata (150 mg/L) (Ferrari et al. 2003). This low LOEC value indicates that this microbioassay could detect levels of this pharmaceutical in river waters with concentrations above 10^{-3} mg/L.

Our microbioassay is not only highly sensitive and fast, but also comes to satisfy the need for efficient environmental monitoring of terrestrial ecosystems (Dominguez-Morueco et al. 2014). Furthermore, thanks to this microbioassay, it would be possible to develop a user-friendly kit to evaluate in situ the toxicity of a sample. Given the standard excitation and emission wavelengths of both biomarkers used, they can be measured in standard fluorometers and even in portable fluorometers. Environmental waters, soil leachates and even air sample water extractions can be assessed. The results of our rapid microbioassay, which takes 5 min, presents advantages compared standardized protocols such as Microtox®, *Vibrio fisheri*'s bioluminescent bacterial bioassay, with contact times from 5 to 15 min (Sandín-España et al. 2013) whose biological and ecological relevance is controversial regarding eukaryotes.

5 Conclusions

The rapid microbioassay using the phycobiont *A. erici* developed in this work shows great advantages. This aero-terrestrial microalga isolated from lichens is representative of terrestrial ecosystems, which unlike aquatic systems, are in urgent need for cost-efficient massive testing methods. Our results suggest that the impact of certain pollutants may be very different on aquatic and aero-terrestrial microalgae, while the former seem more sensitive to heavy metals, the latter are affected in a greater deal by clofibric acid. The culture of the cells on treated paper, besides being a biologically relevant presentation of this organism, allows easy manipulation and transport for laboratory or in situ testing. Especially after dehydration. Both biomarkers used provide relevant information about toxicity, however, free radical content reported by $DCFH_2$-DA is several orders of magnitude more sensitive than chlorophyll autofluorescence. The standard excitation and emission wavelengths allow adaptation to most fluorometers, even portable devices.

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Author's contributions The study was designed by MC. The experiments were performed by MRH. Both authors wrote and revised the texts.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Abbreviations $DCFH\text{-}DA_2$, Dichlorodihydrofluorescein diacetate; DCF, Dichlorofluorescein; LOEC, Lowest Observed Effect Concentration; NOEC, No Observed Effect Concentration; ROS, Reactive oxygen species

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