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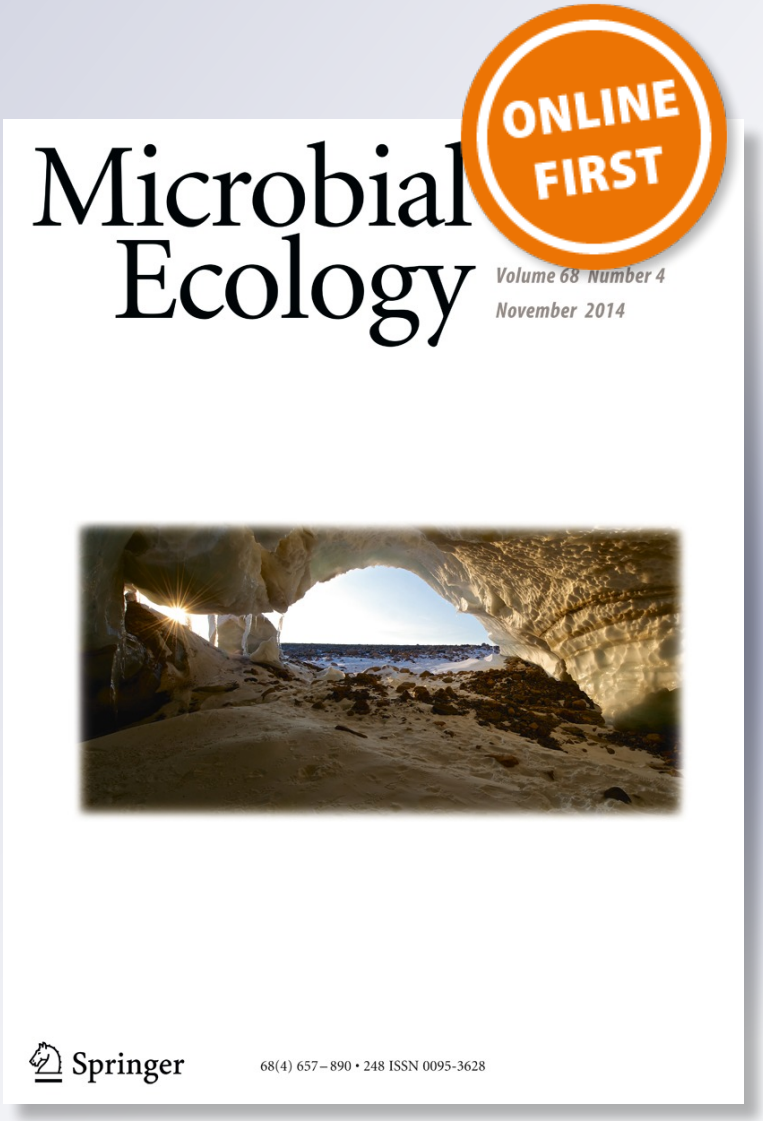

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Lichen Rehydration in Heavy Metal-Polluted Environments: Pb Modulates the Oxidative Response of Both *Ramalina farinacea* Thalli and Its Isolated Microalgae

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Abstract Lichens are adapted to desiccation/rehydration and accumulate heavy metals, which induce ROS especially from the photobiont photosynthetic pigments. Although their mechanisms of abiotic stress tolerance are still to be unravelled, they seem related to symbionts' reciprocal up-regulation of antioxidant systems. With the aim to study the effect of Pb on oxidative status during rehydration, the kinetics of intracellular ROS, lipid peroxidation and chlorophyll autofluorescence of whole *Ramalina farinacea* thalli and its isolated microalgae (*Trebouxia* TR1 and *T.* TR9) was recorded. A genetic characterization of the microalgae present in the thalli used was also carried out in order to assess possible correlations among the relative abundance of each phycobiont, their individual physiological responses and that of the entire thallus. Unexpectedly, Pb decreased ROS and lipid peroxidation in thalli and its phycobionts, associated with a lower chlorophyll autofluorescence. Each phycobiont showed a particular pattern, but the oxidative response of the thallus paralleled the TR1's, agreeing with the genetic identification of this strain as the predominant phycobiont. We conclude that: (1) the lichen oxidative behaviour seems to be modulated by the predominant phycobiont and (2) Pb evokes in *R. farinacea* and its phycobionts strong mechanisms to neutralize its own oxidant effects along with those of rehydration.

Keywords Heavy metal · Lichen · Microalga · Rehydration · ROS · *Trebouxia*

Introduction

Lichens are symbiotic associations that involve at least two very different organisms, a heterotrophic fungus (mycobiont) and a photosynthetic partner or photobiont, which can be either cyanobacteria or unicellular green algae. The lichen symbiosis involves close morphological and physiological integration between mycobiont and photobionts giving rise to the lichen thallus, which is a unique entity or holobiont [30]. Despite this, we ignore how they recognize each other, organize within the thallus, contribute to biological functions or communicate to respond to the environment. Lichens are poikilohydric organisms, such that their water content is mainly determined by the availability of water in the environment. In the desiccated state, water accounts for only 10–20 % of the fresh weight of thalli [6]. While this level of dehydration would be lethal for most organisms, most lichens are desiccation tolerant, surviving in a state of suspended animation until water again becomes available, which allows them to resume their normal metabolism [17, 23, 40].

Desiccation/rehydration processes result in increased levels of reactive oxygen species (ROS) such as: superoxide radicals, hydroxyl radicals, singlet oxygen, hydrogen peroxide and free organic radicals derived from them, especially in

M. Catalá and L.M. Casano have contributed to the same extent in this work.

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photosynthetic organisms [15]. ROS are normal by-products of several metabolic pathways such as photosynthesis and respiration. In general, it is considered that at low concentrations, ROS play a role in cell signalling and defence responses, but at relatively high rates of ROS formation, oxidative damage arises since ROS and ROS-derivative levels exceed the capacity of the antioxidant protection system [24]. ROS can provoke widespread cellular damage by causing pigment and protein degradation, lipid peroxidation and DNA alterations ([41] and cites therein). In lichens, both desiccation and rehydration occur rapidly due to the absence of a waxy cuticullar barrier to water exchanges, making both processes potentially more harmful than in plants. For example, some desiccation-tolerant vascular plants ("resurrection plants") degrade their chlorophyll during dehydration and resynthesize it after rehydration [31], thus preventing unbalanced ROS production. However, lichen photobionts do not significantly alter their content of photosynthetic pigments during their rapid and cyclic changes in water content. Therefore, the ROS formation rate is potentially enhanced in these organisms during desiccation and/or rehydration because CO_2 fixation is impaired, whereas light continues to be absorbed by chlorophyll and electrons transported through redox intermediates to O_2 [6]. Another important metabolic source of ROS is the respiratory chain, thus potentially involving both symbiotic partners. In mitochondria, rapid changes in water content can disrupt normal function and increase ROS formation, especially of superoxide anion radicals [21]. According to Beckett et al. [6] lichens would have evolved different ways of uncoupling the respiration electron flow from phosphorylation to dissipate energy as heat thus preventing an unbalanced formation of ROS. This contention tends to minimize the contribution of the mycobiont to the overall increase in ROS levels during lichen desiccation-rehydration. Accordingly, we have observed that the main sources of intracellular ROS are the phycobiont cells in rehydrating thalli of *Ramalina farinacea* [10].

In addition, the absence of surface protection barriers means that the lichen thalli are highly permeable to airborne substances, including both essential mineral elements and highly toxic pollutants such as heavy metals (HMs). They are among the most valuable biomonitors of environmental pollution both for inorganic and organic compounds, being able to accumulate mineral elements far above their needs [2, 3, 32, 41]. As considered in more detail in a previous study [1], there is a vast variability in HM-tolerance among lichens. However, the physiological basis of such tolerance and especially the relative contribution of the mycobiont and photobionts to the HM-tolerance of the holobiont is still a matter of debate [1]. Anyway, the intracellular accumulation of HMs is a potential metabolic risk for lichens. Heavy metals—and Pb in particular—increase the production of ROS [27, 33, 38]. One of the physiological processes which is highly sensitive to Pb is photosynthesis, probably due to the strong detrimental effects of ROS on some critical components of the photosynthetic machinery. According to Sharma and Dubey [39] Pb inhibits photosynthesis by causing multiple disorders at both structural and biochemical levels, compromising the thylakoid ultrastructure, the pigment and lipid composition and photochemical activities in plants. In addition, Pb inhibits the carboxylase activity of RuBisCO and probably other enzymes of the Calvin cycle due to its direct interaction with –SH groups, or indirectly via Pb-induced ROS which oxidize protein –SH groups [33]. In lichens, these detrimental effects on photosynthesis lead to a progressive increase in oxidative membrane damage [8], at least in the short term, while in the long term, algal survival, and that of the entire holobiont, could be affected.

The lichen *R. farinacea* (L.) Ach. is a worldwide-distributed fruticose, epiphytic lichen with large environmental tolerance. In the Iberian Peninsula, it occurs at all altitudes, more frequently in areas with regular foggy weather, showing preference for places with high atmospheric humidity. This lichen can be found in polluted and unpolluted areas [16, 28, 29] indicating, at least, a moderate-high tolerance to Pb, as suggested by previous experiments in the laboratory [8]. Recently, we have demonstrated that each thallus of *R. farinacea* contains not just one, but two *Trebouxia* phycobionts (provisionally named TR1 and TR9). Furthermore, they show distinct ultrastructural features and physiological responses to acute oxidative stress [9, 14]. In addition, when these phycobionts were incubated in a liquid medium containing Pb, the TR9 algae were able to limit the entry of the toxic metal into the cell, by holding it in the form of aggregates on the outer layer of the cell wall. Under the same conditions, TR1 had a lower level of wall Pb retention capability [1]. Conversely, the intracellular Pb uptake was approximately three times higher in TR1 than in TR9. However, neither the photosynthetic pigment content nor the main photosynthetic electron transport parameters were strongly affected by the presence of Pb within cells in either TR1 or TR9. Control levels of important antioxidant enzymes such as glutathione reductase, superoxide dismutase and ascorbate peroxidase were significantly higher in TR1 than in TR9. However, Pb induced the three enzymes in TR9 while it had no effect on TR1, so that antioxidant activities were quantitatively similar in both phycobionts under Pb treatments. It seemed that each algal species may have developed a specific strategy for the acquisition of similar levels of Pb tolerance [1].

The mechanisms allowing lichens to survive extreme abiotic stress, including the relative contribution of each symbiotic partner, are still unknown. The physiological free-radical burst observed during lichen rehydration [10] could be seriously aggravated by the presence of HM, and the oxidative balance overcome, the photobiont's photosynthetic pigments being the critical targets. Therefore, we hypothesized that

R. farinacea subjected to desiccation/rehydration processes in the presence of Pb would synergistically increase ROS production, especially during rehydration, when algal chlorophyll recovery takes place more rapidly than the CO_2 fixation cycle. Nevertheless, lichens seem to be able to cope with both high Pb levels and desiccation/rehydration cyclic stress. There is evidence of effective communication between mycobionts and photobionts, in which one partner upregulates the antioxidant system of the other, endowing the symbiotic association with an important adaptive advantage and evolutionary success [11, 25] and, as stated above, different photobionts within the same thallus could provide different mechanisms of tolerance to Pb. In consequence, the objective of the present study was to analyse the effects of rehydration in the presence of lead on the kinetics of intracellular ROS production, oxidative damage of membranes and chlorophyll autofluorescence in thalli of *R. farinacea* as compared with its isolated *Trebouxia* phycobionts. The clarification of the individual role of each symbiont in abiotic stress tolerance and damage could help both prevent pollution-led lichen biodiversity loss and the identification of potential biotechnological tools.

Material and Methods

Chemicals

The chemicals 2,6-di-tert-buthyl-4-methylphenol trichloroacetic acid (BHT), 2-thiobarbituric acid (TBA) and 1,1,3,3,tetraethoxypropane were provided by Sigma-Aldrich Química S.A (Tres Cantos, Spain); 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), hydrochloric acid (HCl) and ethanol (etOH) were purchased from Panreac Química S.A.U (Barcelona, Spain) and lead nitrate $Pb(NO_3)_2$ was provided by Merck KGaA (Darmstald, Germany).

Lichen Material

R. farinacea (L.) Ach. was collected in the air-dried state from *Quercus rotundifolia* Lam. at El Escorial (Madrid, Spain; 40°34′54.3′N 4°07′35.1″W) or at Sª El Toro (Castellón, Spain; 39°54′16″N, 0°48′22″W). Samples were completely desiccated in a silica gel atmosphere for 24 h and fragmented into small pieces (ca. 30 mg FW) and frozen at –20 °C until the experiments took place, 1 month after collection.

DNA Isolation, Amplification and Sequencing

Thalli from El Escorial population were surface-washed by immersion for 10 min in aqueous sodium hypochlorite (final concentration 3 % *w*/*v*) and then with sterile distilled water prior to DNA isolation. Total DNA was extracted from 5 mg of dried material using the DNeasy Plant Mini Kit (Qiagen GmbH, D-40724 Hiden Germany) following the manufacturer's instructions. Fungal nrITS were amplified using the primers ITS1F [18] and ITS4 [43]. Algal nrITS were PCR-amplified using the primers of [26]. A portion of the plastid 23SrDNA was PCR-amplified using the primers 23Sps1 5′-CAT GAC CAG GAT GAA GCT TG -3′, 23Sps2 5′- TAC TCA AGC CGA CAT TCT C -3′ and cL781R [9]. Amplification products were sequenced as previously described [13].

Phycobiont Isolation and Culture

Trebouxia TR1 and TR9 were isolated in our laboratories from specimens of the populations mentioned above, according to the method of Gasulla et al. [20]. TR1 and TR9 phycobionts were grown on small nylon discs (5-mm diameter) on semisolid Bold 3N medium [7] containing 10 g casein and 20 g glucose per litre [4] at 15 °C, under a 14-h/10-h light/dark cycle (lighting conditions 25 μmol m^{-2} s^{-1}) in a growth chamber.

Rehydration Treatments in Presence of Lead

After 21 days, the discs (20–30 mg cell fresh weight/disc) were removed from the culture medium and subjected to slow desiccation in a closed container with a saturated solution of K_2SO_4 (80 % relative humidity) for 24 h. Other environmental conditions were 20 °C, a 14 h/10-h light/dark cycle (2–5 μmol m^{-2} s^{-1}).

Both the desiccated phycobiont cultures and fragments of lichen thalli were placed in black flat bottom 96-multiwell plates and rehydrated with (100–150 μL) of either 0, 50 or 100 μM $Pb(NO_3)_2$ in water. After 5 min, the excess solution was discarded.

Fluorometric Kinetics of Free Radical Production and Chlorophyll Autofluorescence

2,7-Dichlorodihydrofluorescein diacetate was used as a probe in the detection of free radicals [42] (DCF, λ_{exc}=504 nm, λ_{em}=524 nm, green) during the rehydration of both desiccated lichen thalli and isolated phycobionts in the presence of lead. $DCFH_2$-DA is not appreciably oxidized to the fluorescent state without prior hydrolysis inside the cell. At the same time, we followed the physiological state of the chlorophylls by analysing the autofluorenece of these pigments in the same samples.

For this purposes, 10 μM $DCFH_2$-DA was added to the lead solutions employed for rehydration of both lichen thalli and isolated TR1 and TR9, and the kinetics of DCF and chlorophyll-emitted fluorescence were simultaneously measured in a SPECTRAFluor Plus microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Excitation of both

substances was performed at λ_{exc} 485 nm, emission of DCF fluorescence was recorded at λ_{em} 535 nm and chlorophyll autofluorescence at λ_{em} 635 nm, for 3 h. Twelve replicates were analysed by treatment and all values are referred to the weight of sample.

Lipid Peroxidation

Lipid peroxidation was evaluated as thiobarbituric acid reactive substances (TBARS) using Reilly and Aust's method [35], modified as described [10]. Briefly, both fragments of lichen thalli and isolated phycobionts were rehydrated in the presence of lead as described. At indicated times, thalli were weighed and homogenized on ice with deionized water and centrifuged and the pellets re-suspended in 500 μL ethanol containing 2,6-di-tert-buthyl-4-methylphenol (BHT). Algae were weighed and homogenized directly in 500 μL of ethanol-BHT. Subsequently, 900 μL of 257 mM TBA, 0.918 M tri-chloroacetic acid and 3.20 M HCl were added to each sample and to the standard (1,1,3,3,tetraethoxypropane), which were incubated at 70 °C for 30 min and centrifuged. The absorbance of supernatants was measured at 532 nm (A_{532}) and 600 nm (A_{600}). This last value was subtracted from the A_{532} to eliminate interferences.

Statistics

At least six independent lichen samples and 12 isolated phycobiont cultures for each treatment were prepared. Four assays were carried out on four different days for the lichens and on three different days for the algae. Data were analysed for significance with Student's *t* test or by ANOVA.

Results and Discussion

Molecular Characterization of *R. farinacea* Population from El Escorial (Madrid, Spain)

In previous studies, we analysed the genetic structure of diverse populations of *R. farinacea* from the Iberian Peninsula, the Canary Islands and California [9, 13]. Morphological and genetic evidence indicated that all the analysed thalli contained both *Trebouxia* TR1 and *T.* TR9 phycobionts at the same time. However, TR1 seemed to be "predominant" or relatively more abundant, while TR9 were the "non-predominant" phycobionts in all samples except in thalli collected from the Canary Islands. Therefore, thalli are heterogeneous and the relative abundance of each symbiont is not constant but may vary depending on the population used (the underlying mechanisms being unknown). In the present study, we employed thalli from a new *R. farinacea* population, collected at El Escorial (Madrid) in the central region of Spain. This new lichen population was characterized with the aid of mycobiont- and phycobiont-specific moleclular markers with the following purposes: (1) to corroborate the morphological identification of collected thalli, (2) to determine the probable coexistence of TR1 and TR9 phycobionts in each thallus and estimate their relative abundance and (3) to assess possible correlations among the relative abundance of each phycobiont, their individual physiological responses and that of the entire thallus.

Total DNA was extracted from a randomized sample of 12 thalli. The correct classification of the sample as *R. farinacea* was confirmed for six randomly selected thalli on the basis of the nrITS as described in [13] (accessions: KJ413040, KJ413041, KJ413042, KJ413043, KJ413044, KJ413045). BLAST searches against nucleotide sequences from the NCBI showed that the fungal partner effectively corresponded to *R. farinacea*. To test the identity of phycobionts, a portion of the chloroplast 23SrDNA was amplified with primers 23Sps1 and 23Sps2 (Fig. 1a). DNAs from all thalli rendered a band of ca. 0.6 Knt (Fig. 1b), which is in agreement with the expected size of 23Sps1/23Sps2 amplicons from TR1 algae (Fig. 1a). The sequence of six randomly selected bands was shown to be

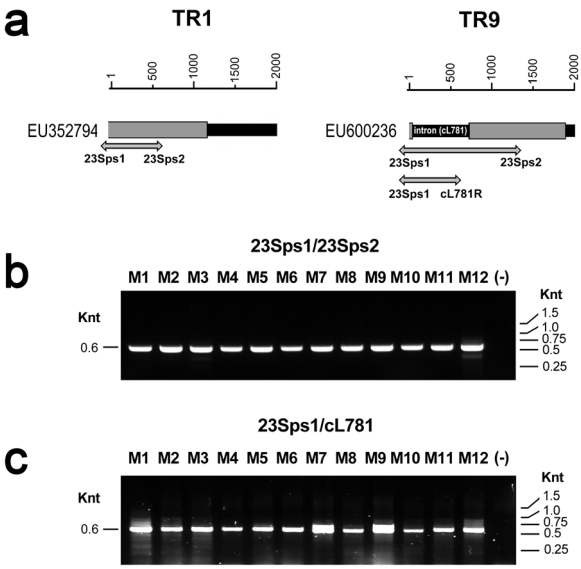


Fig. 1 Molecular characterización of mycobiont and microalgae of *Ramalina farinacea* thalli from El Escorial (Madrid, Spain). **a** Genetic maps of the sequenced portion of plastid LSU rDNA in *Trebouxia* TR1 and *T.* TR9 phycobionts. The *grey boxes* represent exons. Introns are depicted as *black lines* between exons. Only the name of the cL781 intron [24] is indicated. The expected amplification products and primer names are indicated below each map with *light-grey arrows*. **b** Gel electrophoresis of PCR amplification products obtained with the 23Sps1/23Sps2 primer pair. **c** Gel electrophoresis of PCR amplification products obtained with the 23Sps1/cL781R primer pair. Amplifications were performed with DNA extracted from 12 thalli (M1 to M12) collected at El Escorial (Madrid, Spain). Electrophoreses were performed in 2 % agarose gels. Negative controls (−) correspond to PCR reactions without DNA. The sizes of molecular markers, and the expected products, are indicated on the *right* and the *left*, respectively

identical, or similar to previously published sequences for TR1 phycobionts [13, 12]. To test the co-existence of TR9 in all thalli as previously demonstrated in [9], we used the 23Sps1 and cL781R primer pair (Fig. 1a). Primer cL781 amplifies DNA from TR9 algae (harbouring the cL781 intron) but not from TR1 algae (lacking the cL781 intron). As shown in Fig. 1c, all samples rendered amplification products of the expected size (0.6 kb). Sequencing of these bands confirmed their correspondence to TR9 phycobionts.

It is noteworthy that when "universal primers" for the nrITS of *Trebouxia* algae were used, a single algal nrITS sequence per thallus was obtained for each of them, which clearly corresponded to TR1 phycobionts (accessions: KJ413046, KJ413047, KJ413048, KJ413049, KJ413050, KJ413051). Accordingly, this algal species was taken to be the "predominant phycobiont." The less abundant was detected only by using specific primers (23Sps1/cL781R) and corresponded to TR9. These results agree with previous studies [9, 12]. In summary, the identity of symbionts of *R. farinacea* from the El Escorial corresponds to that of other Mediterranean-type populations of this lichen in which *Trebouxia sp*. TR1 is the "predominant" phycobiont.

Effect of Pb on Oxidative Status of ***R. farinacea*** and Chlorophyll Autofluorescence During Rehydration

Previously, we have demonstrated, by employing the fluorescent probe $DFCH_2$-DA, that *R. farinacea* thalli significantly increased intracellular ROS production during the first 45 min of rehydration [10, 11]. In those studies, the fluorometric kinetics of intracellular ROS production in rehydrating lichen thalli showed that the rate of intracellular free radical production continuously increased, reaching values 10-fold higher at 45 min with respect to those at the beginning of rehydration (see Fig. 3A and 3b in [10]). In the present experiments, we have subjected *R. farinacea* thalli to rehydration with water containing 0 (control) and 100 μM $PbNO_3$ and the kinetics of intracellular ROS production was followed up to 165 min employing the fluorescent probe $DFCH_2$-DA, with the same methodology as used in the studies referred to above. The presence of lead in the rehydration solution had a strong impact on the intracellular free-radical formation (Fig. 2a), especially during the first 90 min of rehydration when the HM not only significantly and progressively decreased the level of ROS with respect to control values but also changed ROS release kinetics. From 90 min on, the intracellular ROS level was maintained in less than 40 % of control values in Pb-rehydrated thalli. It seems that Pb elicited a defensive mechanism known as "hormetic response," which in this case would involve, at least in part, the activation of free-radical scavenging or an antioxidant defence system [34]. However, it should be noted that the absolute amount of ROS continuously increased by 10-fold in the first hour of rehydration, reaching a 15-fold increase after 165 min of rehydration (Fig. 2a). Therefore, absolute ROS levels in Pb-rehydrating thalli were kept ca. 4-fold higher with respect to their own initial values (Fig. 2a).

Lipids in general are within the main targets of ROS [37], and particularly, their fatty acids undergo a self-propagating oxidative damage called lipid peroxidation, which can be assessed through some metastable products capable of reacting with thiobarbituric acid (TBARS). According to the results shown in Fig. 2b, the presence of lead during rehydration did not significantly affect the pattern of TBARS formation with respect to that of the control. It is possible that the remnant ROS level in Pb-rehydrated thalli of *R. farinacea* could be sufficiently high to cause the same extent of lipid peroxidation as that observed in water-rehydrated ones. Another physiological parameter related with the oxidative damage of membranes and the integrity and functionality of the photosynthetic apparatus is chlorophyll autofluorescence, which was simultaneously recorded during the rehydration of the thalli. As can be observed in Fig. 2c, chlorophyll autofluorescence in water-rehydrated thalli sharply increased during the course of rehydration. The presence of 100 μM $PbNO_3$ diminished chlorophyll autofluorescence to ca. 80 % with respect to the control values for the first 75 min of rehydration and to ca. 60 % afterwards, indicating a delay in the reorganization of the light-capturing system. Since the photosynthetic electron chain is one the main sites of ROS generation [14 and cites therein], the observed decrease in chlophyphyll autofluorescence in Pb-rehydrated thalli could be causally linked to the lower rate of intracellular free radical production.

Intracellular ROS Formation and Oxidative Parameters During Rehydration in Isolated Phycobionts of ***R. farinacea***

The *Trebouxia* phycobionts (provisionally TR1 and TR9) coexisting in *R. farinacea* thalli showed distinct physiological responses to light intensity, temperature and oxidative stress generated by the ROS propagator cumene hydroperoxide [9, 14]. In addition, liquid cultures of TR1 and TR9 were similarly tolerant to lead exposure, but employed different morpho-physiological strategies against the heavy metal [1]. In the present study, we characterized the kinetics of intracellular oxygen free-radical formation and two related oxidative parameters (TBARS and chlorophyll autofluorescence) during rehydration, initially with water and then in the presence of two concentrations of $PbNO_3$, in isolated and previously desiccated TR1 and TR9 phycobionts. We employed 50 and 100 μM $PbNO_3$ treatments, the latter is the same as that used in studies of thalli rehydration, and the former simulates a possible "biosorption effect" effect of the mycobiont which might limit the amount of HM which accesses the phycobiont cells. The objectives of these experiments were to search for

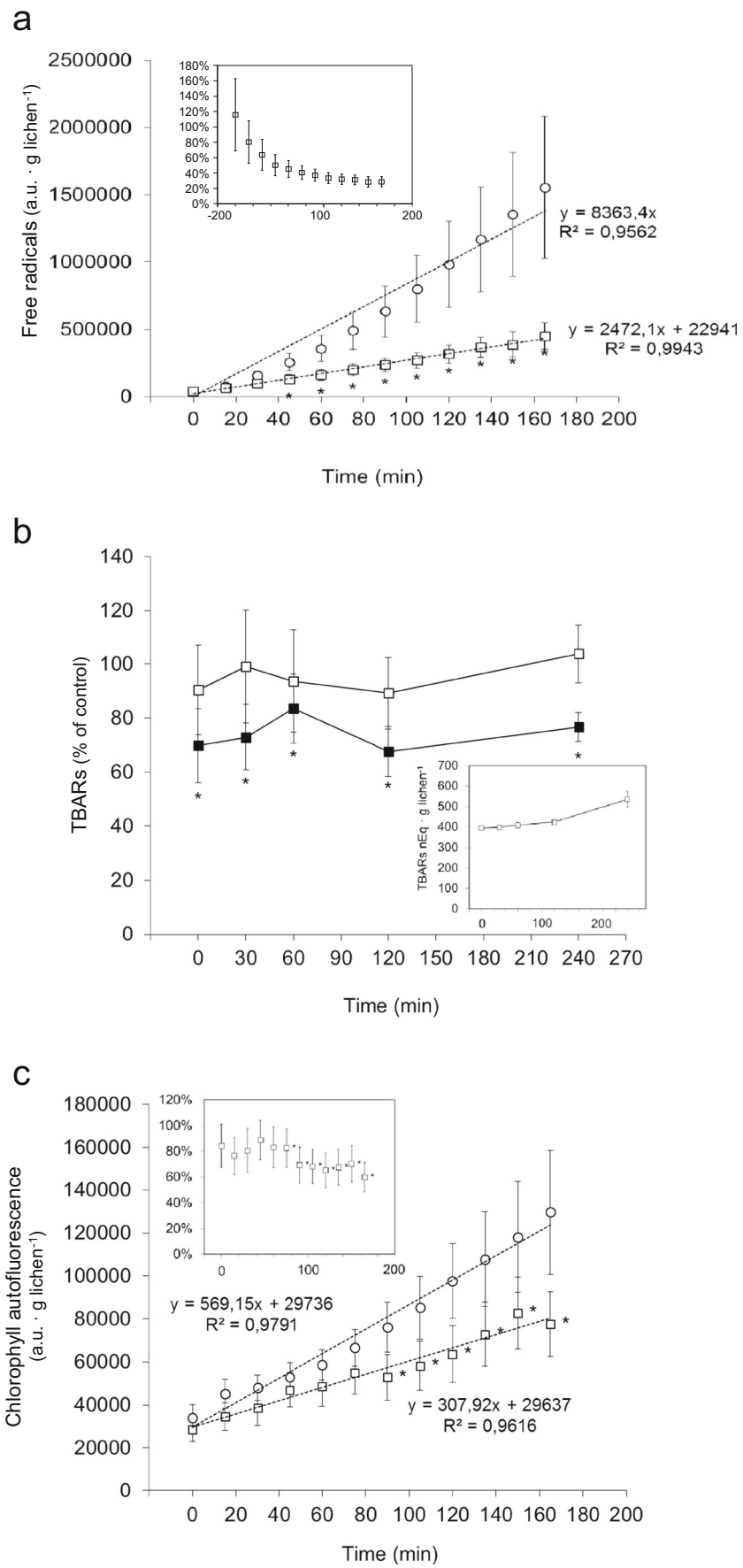
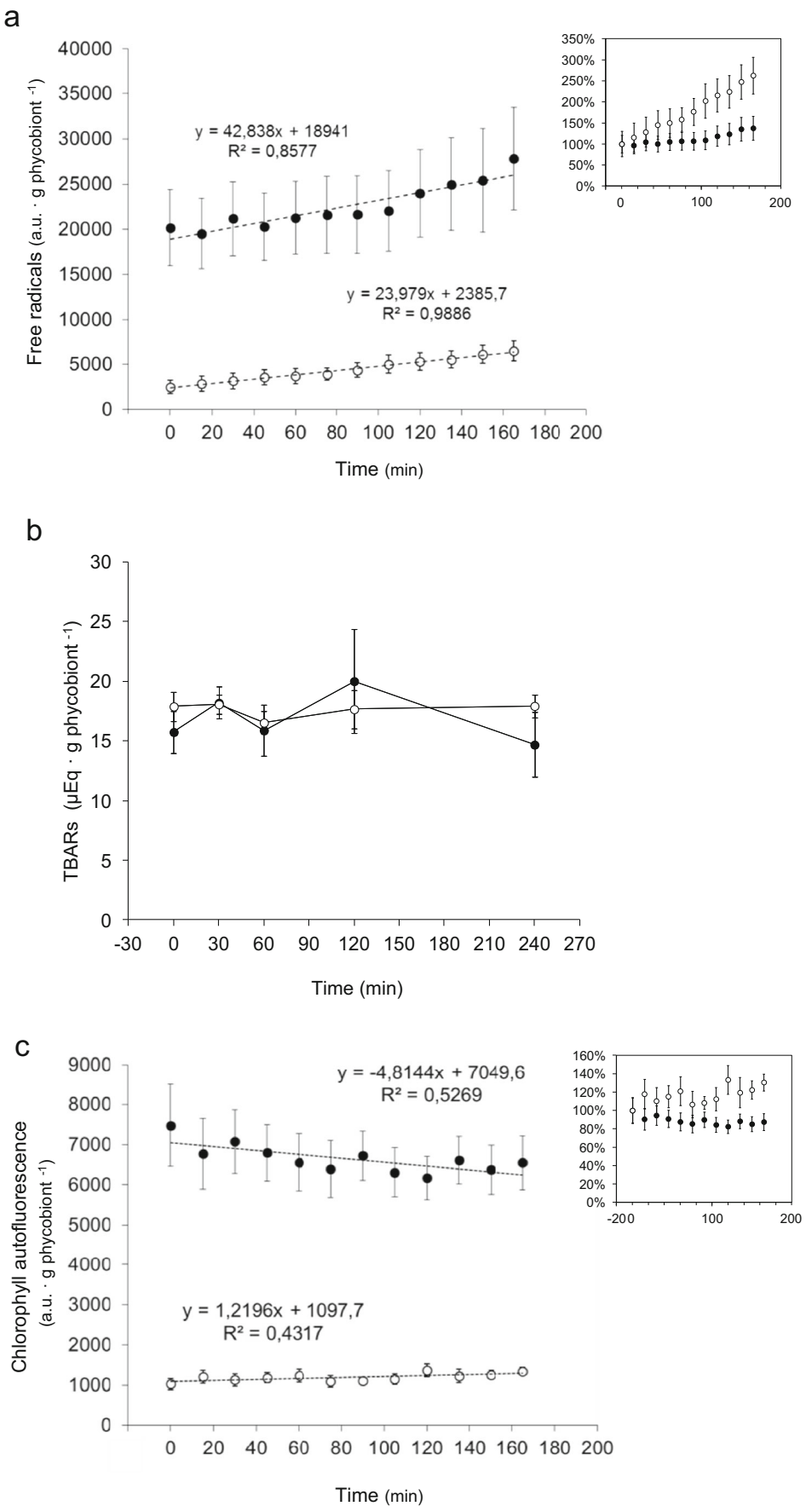


Fig. 2 Effects of Pb on the oxidative parameters of *Ramalina farinacea* thalli during rehydration. Kinetics of intracellular free-radical production (**a**) and chlorophyll autofluorescence (**c**) in thalli rehydrated with deionized water (*white circle*) or 100 μM $PbNO_3$ (*white square*). Fluorescence units are arbitrary and comparisons of relative magnitudes can only be made within the same graph. Insets in (**a**) and (**c**) show the intracellular free radical and chlorophyll autofluorescence levels referred to controls, respectively. (**b**) TBARS content referred to controls in thalli rehydrated with either 50 μM (*black square*) or 100 μM $PbNO_3$ (*white square*). Statistical test (referred to control): $*p<0.05$. The inset shows the TBARS content [$nEq.g(FW)^{-1}$] of thalli rehydrated with deionized water. Symbols represent means and standard error bars (SE) of 12 or at least 6 replicates, for (**a**) and (**c**) or (**b**), respectively

possible differences in the physiology of rehydration in the absence/presence of Pb between the two phycobionts and to assess the influence of each alga on the overall behaviour of the entire holobiont.

As shown in Fig. 3a, TR1 and TR9 showed distinct patterns of intracellular ROS formation from both a quantitative and a qualitative perspective. Indeed, while free radicals linearly increased during the course of rehydration in isolated TR9 cells, in TR1, the level of these compounds remained almost unchanged for the first 105 min; thereafter, it evidently increased by ca. 40 % with respect to the initial values at 165 min of rehydration. Moreover, it is noteworthy that rehydrating cultures of TR1 always produced remarkably more free radicals than TR9. The relative differences in intracellular ROS levels between TR1 and TR9 ranged from ca. 6.5- to 4.5-fold at the beginning and the end of the studied period of rehydration, respectively. Interestingly, similar quantitative differences in chlorophyll autofluorescence between the two phycobionts of *R. farinacea* were observed (Fig. 3c). This parameter did not significantly change during rehydration in both algae, but it was always ca. 6.5-fold higher in TR1 than in TR9. On the other hand, the great differences in free-radical production between the two algae seemed not to have any correlative impact on lipid peroxidation, since the amount of TBARS was similar in both TR1 and TR9 and during the course of rehydration (Fig. 3c). These quantitative discrepancies could be explained, at a first glance, by putative differences in ROS-scavenging differences between TR1 and TR9 algae. However, in previous studies [1, 14], we have observed that even though basal levels of antioxidant enzymes glutathione reductase, superoxide dismutase and ascorbate peroxidase were significantly higher TR1 than in TR9 (liquid cultures), Pb treatments induced the three enzymes in TR9 while it had no significant effect on those of TR1. In consequence, antioxidant activities were quantitatively similar in both phycobionts under Pb treatments [1]. Perhaps, other still-undetermined antioxidant agents could be responsible, at least in part, for the observed discrepancies between quantitative levels of ROS formation and lipid peroxidation. On the other hand, one have to keep in mind that results are expressed on cell mass basis and thylakoid membrane lipids are one of the main sources of lipid peroxides in algal and plant cells. Previous ultrastructural analysis by TEM of TR1 and TR9 algae indicated great differences in thylakoid density within their chloroplast [9]. In that article, we described: "In TR1 cells, thylakoids were often closely associated in stacks of three at the most …In TR9 cells, …thylakoids were grouped in stacks shaped by numerous membranes, similar to the grana in vascular plants." In addition, "large spherical vesicles with an electron-dense content made up of lipids were seen throughout the cytoplasm (of TR9 but not of TR1) and were especially abundant at the periphery and near the mitochondria". It is thus possible that morphological and biochemical differences could lead to differences in the total amount of lipids/cell between TR1 and TR9, being relatively lower in the former than in the latter. This fact could be of crucial importance in determining the amount of lipid peroxides/cell, since lipid peroxidation is a self-propagating process and only needs the direct participation of ROS at the starting reaction(s) [36].

When rehydration was performed in the presence of aqueous solutions containing either 50 or 100 μM $PbNO_3$, significantly different physiological responses could be observed in each phycobiont (Figs. 4 and 5). TR1 algae diminished the intracellular ROS formation, but followed different time-dependent patterns depending on the concentration of the HM (Fig. 4a). At 50 μM $PbNO_3$, this phycobiont showed a slow but progressive reduction of free radicals reaching ca. 40 % with respect to control values at the end of rehydration. When rehydrated in the presence of 100 μM Pb, TR1 sharply lowered ROS levels to 40–45 % with respect to control values at 30 min of rehydration. However, either 50 or 100 μM $PbNO_3$ induced quite the opposite effect on intracellular ROS production during rehydration in TR9 (Fig. 5a). Under a lower level Pb treatment, this alga was able to decrease the intracellular free radicals to ca. 50 % with respect to control values almost from the beginning to the end of rehydration. In contrast, under 100 μM Pb, TR9 could only maintain ROS production at similar levels, or slightly higher (but not significantly) than those of controls during the initial 105 min, thereafter free radicals augmented by ca. 40 %. The effects of lead during rehydration on the kinetics of chlorophyll autofluorescence of isolated TR1 and TR9 clearly resembled those observed on intracellular ROS formation (Figs. 4c and 5c, and Figs. 4a and 5a, respectively). TR1 cells diminished chlorophyll autofluorescence by ca. 50 and 40 % with respect to controls when rehydrating in the presence of 50 and 100 μM Pb, respectively. However, TR9 either decreased or increased this photosynthetic pigment parameter if the concentration of lead during rehydration was either 50 or 100 μM $PbNO_3$, respectively, in proportions similar to those observed for free-radical production. As shown in Figs. 4b and 5b, the presence of lead provoked a significant and transient diminishing of lipid peroxidation in both phycobionts at 30 min of rehydration. However, later on, dose- and time-dependent differences between the two algae were observed. When rehydrated with 50 μM Pb, TR1 did



◀ **Fig. 3** Rehydration of desiccated cultures of *Trebouxia* TR1 and *T.* TR9 microalgae isolated from the lichen *Ramalina farinacea*. Kinetics of intracellular free radical production (**a**) and chlorophyll autofluorescence (**c**) [a.u.·g(FW)$^{-1}$] in either TR1 (*black circle*) or TR9 (*white circle*) rehydrated with deionized water. Insets in (**a**) and (**c**) represent the intracellular free radical and chlorophyll autofluorescence levels referred to their corresponding initial values, respectively. (**b**) TBARS content [nEq.g(FW)$^{-1}$] in either TR1 (*black circle*) or TR9 (*white circle*) rehydrated with deionized water. *Symbols* represent means and standard error bars of 12 replicates

not modify its levels of TBARS with respect to controls, while under 100 μM Pb, it diminished lipid peroxidation by ca. 30 and 40 % with respect to controls, at 60 and 120 min of rehydration, respectively, even though there was no statistical significance probably due to intraspecific variability (Fig. 4b). Conversely, for TR9, 50 μM Pb seemed to be more effective than 100 μM in maintaining TBARS at lower levels with respect to controls, especially at 2 h of rehydration (Fig. 5b). In the next 2 h, lipid peroxidation tended to smoothly increase in TR1 and TR9 phycobionts exposed to both lead concentrations, which still showed slightly lower levels of TBARS (80–90 %) than controls, but the differences were not statistically significant. On the other hand, there seemed to be a qualitative, or not-strictly quantitative, relationship between the kinetics of intracellular ROS formation and those of TBARS (Figs. 4a and 5a, and Figs. 4b and 5b, respectively). A distinct species-specific lead concentration (100 μM in TR1 and 50 μM in TR9) was the most effective in diminishing both free radicals and lipid peroxidation.

The Effect of Lead During Rehydration of Lichen Thallus in Comparison with Its Isolated Phycobionts. Hormesis

In general, the bulk of phycobiont biomass accounts for ca. 5–7 % of total lichen biomass. More than 90 % corresponds to the mycobiont [5]. However, these proportions do not seem to reflect the relative contribution of each symbiotic partner to the formation of intracellular free radicals. According to previous results from Catalá et al. [10, 11], phycobionts are the major source, and cortical tissue is the second producer of intracellular ROS, from a quantitative perspective. Since, in our experiments, the ROS produced have not been quantified using an external standard, quantitative comparisons of whole thallus and isolated phycobionts' ROS production are not possible. However, the comparison of kinetic patterns and relative ROS variations provide very interesting information. At 165 min of rehydration with water, *R. farinacea* thalli generated an amount of free radicals equivalent to 15-fold from its initial levels,

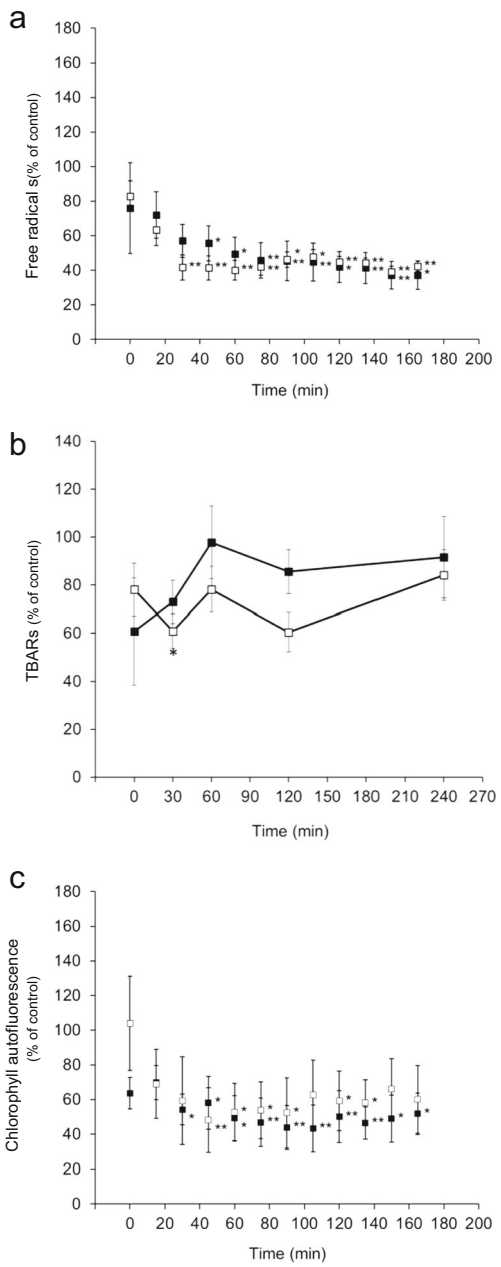


Fig. 4 Rehydration of desiccated cultures of *Trebouxia* TR1 phycobiont in the presence of Pb. Kinetics of intracellular free radical production (**a**), lipid peroxidation (TBARs content, **b**) and chlorophyll autofluorescence (**c**) referred to controls (see Fig. 3) in isolated TR1 phycobionts rehydrated with either 50 μM (*black square*) or 100 μM $PbNO_3$ (*white square*). Statistical test (referred to control): *$p<0.05$; **$p<0.005$. *Symbols* represent means and standard error bars of 12 replicates

while at the same time isolated TR1 phycobionts produced an equivalent of a 40 % increase with respect to the initial value. This effect could be a consequence of differences in the rate of water acquisition during rehydration. This is very fast in desiccated algae (within a few minutes they reach nearly 100 % rehydration [19]), while in thalli, this process can be considerably slower

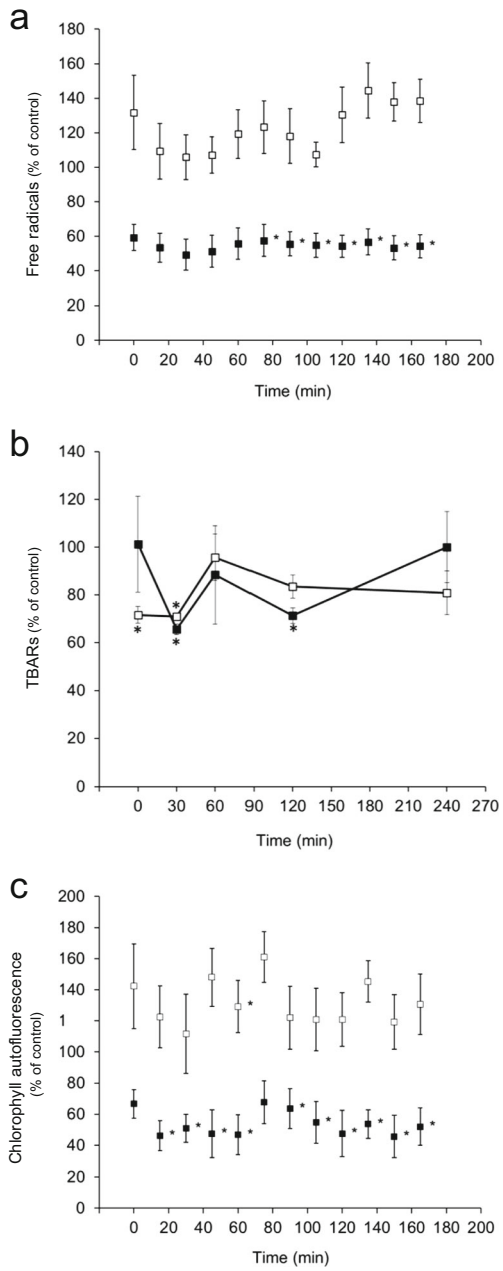


Fig. 5 Rehydration of desiccated cultures of *Trebouxia* TR9 phycobiont in the presence of Pb. Kinetics of intracellular free radical production (**a**), lipid peroxidation (TBARs content, **b**) and chlorophyll autofluorescence (**c**) referred to controls (see Fig. 3) in isolated TR9 phycobionts rehydrated with either 50 μM (*black square*) or 100 μM $PbNO_3$ (*white square*). Statistical tests (referred to control): $*p<0.05$; $**p<0.005$. *Symbols* represent means and standard error bars of 12 replicates

as a consequence of the complex multilayered structure formed by mycobiont hyphae with special water conductance features ([22] and cites therein).

From a qualitative point of view, the comparison of kinetics of intracellular ROS, chlorophyll autofluorescence and lipid peroxidation among the entire thallus and both TR1 and TR9, rehydrated with water alone or in the presence of lead, showed a parallelism between thallus and TR1 (Figs. 2, 3, 4 and 5). This contention agrees with the evidence presented here, indicating that in *R. farinacea* thalli belonging to the El Escorial population, TR1 algae are by far the most abundant, or "predominant" phycobionts, while TR9 algae are the "non-predominant" phycobionts. Moreover, the parallelism between the dynamics of intracellular free-radical formation in the thallus and the predominant phycobiont prompt us to hypothesize that the photosynthetic partner could be an initial source or "starting point" of photoinducible ROS during rehydration. The close contact with mycobiont hyphae would facilitate the migration of diffusible ROS (such as H_2O_2 or other ROS-metastable derivatives) which could induce and propagate new ROS formation within fungal cells. Related to this, it is known that lead damages the photosynthetic membranes due to its affinity for N- and S-ligands: chlorophyll b and thus photosystem II being especially sensitive to Pb toxicity [39]. This toxic effect might be related with the observed diminishing of chlorophyll autofluorescence in both thallus and isolated algae rehydrated in the presence of lead. However, in previous experiments with liquid cultures of TR1 and TR9 exposed to chronic treatments of the same doses of Pb, no evident chlorophyll damage was observed [1]. In consequence, it is reasonable to speculate that when both thallus and isolated phycobionts (TR1 in this case) rehydrate in the presence of Pb, they activate some dissipative mechanism(s) of absorbed energy [19], evidenced as a reduction of chlorophyll autofluorescence, and thus prevent the formation of intracellular free radicals and oxidative stress. In other words, low doses of lead during lichen rehydration could exert a "hormetic" effect [34] by inducing a very fast defensive response of the photosynthetic partners. Interestingly, when desiccated thalli of *R. farinacea* were rehydrated in the presence of 50 μM Pb, they were able to preserve membranes from oxidative damage as indicated by lower lipid peroxidation (Fig. 2b).

Conclusions

The presence of lead during water gain seems to induce, in both *R. farinacea* and its phycobionts, strong mechanisms to prevent the oxidative burst provoked by rehydration and probably strengthened by Pb. These protective mechanisms appear to be very fast and could involve, at least in part, chlorophyll autofluorescence modulations. Our results indicate that the oxidative response of the entire thallus during rehydration seems to be mainly modulated by that of the predominant phycobiont, which can have important ecophysiological and adaptive implications since thalli components proportion may vary from population to population.

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