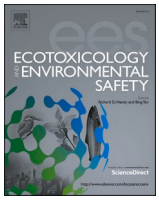
Ecotoxicology and Environmental Safety 229 (2022) 113078

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Biological responses to heavy metal stress in the moss *Leptodictyum riparium* (Hedw.) Warnst

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ARTICLE INFO

Edited by Dr. Caterina Faggio

Keywords:
Leptodictyum riparium
Heavy metal
Photosynthetic yield
Glutathione
Phytochelatin synthesis
Nitrogen metabolism

ABSTRACT

Leptodictyum riparium, a widely distributed aquatic moss, can both tolerate and accumulate very high concentrations of toxic heavy metals, with only slight apparent damage. Here we report the effects on photosynthetic yield, glutathione (GSH), phytochelatin (PCn) synthesis, nitrogen metabolism and cellular localization of molecules rich in SH groups in *L. riparium* exposed in vitro to heavy metals. We simulated the concentrations of Cu, Zn, Cd, Pb detected in Regi Lagni, Italy, one of the most contaminated freshwater sites in Southern Europe, in the laboratory to test how the moss responds to heavy metal contamination. There was a steady decrease of photosynthetic efficiency correlated with the heavy metal concentrations and ultrastructural organization. All PCn levels increased significantly as the concentration of heavy metals increased, while the GSH levels did not appear to be particularly affected. A significant increase of GDH and NADH-GOGAT activities increased with increasing heavy metal concentration. Immunoblotting analysis revealed an increase of the chl-GS2 while no significant increase was detected in the cyt-GS1. These results give insight into the molecular events underlying the metal-tolerance of the aquatic moss *L. riparium* exposed to environmental heavy metal concentrations.

1. Introduction

The aquatic moss *Leptodictyum riparium* (Hedw.) Warnst (*Bryophyta*) can tolerate and accumulate very high concentration of heavy metals (metals that have a specific density of more than 5 g/cm^3), including Cadmium (Cd) (Basile et al., 2012a; Esposito et al., 2018, 2012; Maresca et al., 2018), with bioconcentration levels higher than other plants, including some angiosperms, suffering minimal apparent damage. Due to its tolerance and accumulation capability, *L. riparium* has been chosen as candidate organism for biomonitoring heavy metals in natural environmentals as well as for phytoremediation projects. (Basile et al., 2012b; Esposito et al., 2018; Maresca et al., 2018). Indeed, *Leptodictyum riparium* was among the ten macrophytes that have been proposed for the biomonitoring of toxic metals in European rivers and streams by Say et al. (1981).

Recently, *L. riparium* was used to monitor the state of environmental pollution in two of the most polluted waterways in Campania, Italy; the Sarno river and a system of artificial canals known as Regi Lagni (Esposito et al., 2018; Maresca et al., 2018). The Regi Lagni is a channel system that catches meteoric and waste waters, routing them from the zones sited north of Naples to the Tyrrhenian Sea. The heavy industrialization (i.e., chemical industry, intensive farming) as well as urbanization of the areas near the Regi Lagni resulted in a severe contamination of these water bodies (Bove et al., 2011; Grezzi et al., 2011). Furthermore, the Regi Lagni catchment comprises two zones, the "Land of Fires" and the "Triangle of Death" that have been exploited for illegal waste disposal and contaminated by the ash fallout from uncontrolled garbage incineration which caused dangerous pollution of groundwater and soil (Senior and Mazza, 2004). This severe contamination had a long-term health effect on the local human population,

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https://doi.org/10.1016/j.ecoenv.2021.113078
Received 15 October 2021; Received in revised form 6 December 2021; Accepted 9 December 2021

causing an alarming increase in cerebrum-vascular diseases and cancers (Senior and Mazza, 2004). In an earlier study, *L. riparium* was used to monitor heavy metals pollution in Regi Lagni channels (Maresca et al., 2018). The investigation was carried out installing moss bags in three sites according to a pollution gradient from the less polluted to the more polluted zone. Biological responses such as bioaccumulation of heavy metals, ROS production, antioxidant enzyme activity, DNA damage and HSP70 induction were measured. Heavy metals accumulated in the moss tissues causing severe ultra-structural damages and biochemical responses at higher concentration.

Nevertheless, in natural field conditions the effect of other environmental variables on heavy metal accumulation and potential damage to *L. riparium* , should be investigated. For example, when the chlorophyll fluorescence of moss is used to biomonitor heavy metal contamination in aquatic environments, where both heat stress and high light are present at the same time, the fluorescence parameters are seriously reduced, resulting in inaccurate measures (Chen et al., 2019). Consequently, controlled experiments focused only on the variables of interest, can strengthen the correspondence between the biological responses and the impact of the variables.

To better evaluate the responses of the moss *L. riparium* to heavy metal contamination, and specifically its photosynthetic yield, the presence of glutathione, phytochelatins, nitrogen metabolism, and the cellular localization of molecules rich in SH groups, a controlled experiment was performed. Hence, this study aims to investigate the molecular and biochemical responses of *L. riparium* under controlled conditions, after exposure to external heavy metal sources, comparable to those detected in the three sites of the Regi Lagni (Maresca et al., 2018), in order to improve the knowledge about the tolerance of *L. riparium* to heavy metal stress, and strengthen the idea that this moss could be suitable for biomonitoring in natural field conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Leptodictyum riparium gametophytes were gathered from a water spring in the central zone of the Botanical Garden of the University of Naples "Federico II" (Italy). The gametophytes were carefully washed with deionized water and sterilized with a solution of 7% (v/v) NaClO and a few drops of Triton X-100, then rewashed with deionized water.

Samples were individually put into Petri dishes filled with 25 mL of sterile tap water (control) or heavy metals mix (Cu, Zn, Cd and Pb - concentrations are reported in Table 1S) for total exposure of 7 days. Then gametophytes were exposed in vitro to the same heavy metal concentrations detected in the three stations along the Regi Lagni reported by Maresca et al. (2018) and shown in Table 1S. The samples were cultured in a growth chamber with a temperature in the range of 15 °C ± 1.3 °C / 20 ± 1.3 °C night/day, 70% ± 4% relative humidity RH, 16 h/8 h light/dark cycle and a 40 μmol m^{-2} s^{-1} photosynthetic photon flux density.

To confirm the absence of damage due to the sterilization process, *L. riparium* gametophytes were observed every two days with a Leitz Aristoplan microscope (Leitz,Wetzlar, Germany) and a Wild Heerbrugg M3Z binocular microscope (Leica, Nussloch, Germany) (Bellini et al., 2020). The plant material was grown in triplicate and all the experiments were repeated at least three times.

2.2. Extraction of thiol peptides from L. riparium gametophytes and analysis by HPLC-ESI-MS-MS

Samples of *L. riparium* gametophytes were extracted according to a previously published method (Bellini et al., 2019) with some modifications. Briefly, the extracts instead of being filtered by a Minisart RC4 0.45–μm filter (Sartorius, Goettingen, Germany) were ultrafiltered through Amicon® Ultra (10 K device) centrifugal filters (Merk, Germany) at 14000 g, 4 °C for 30 min and samples were stored at − 80 °C until analysis. The HPLC-ESI-MS-MS analyses were all performed by the instrument layout and the procedure described in Bellini et al. (2019).

2.3. Confocal laser imaging

Samples treatment was according to Maresca et al. (2020a). In short, phylloids were stained with 100 μM MCB (Thermo Fisher Scientific, MA, USA) for 30 min at 21 °C in the dark, at near neutral pH conditions. After, observation was performed under a Leica TCS SP5 confocal laser scanning microscope (CLSM) with a 40X immersion objective. Excitation of MCB and chlorophyll was set at 405 nm wavelength, and emission detected at 460–520 nm (begin-end) and 630–700 nm (begin-end), respectively. Detector gain and offset values were kept fixed to compare the different results.

MCB stock was made as a 50 mM solution in dimethyl sulfoxide (DMSO). A 100 μM solution was prepared adding sterile water to dilute the stock solution. Unstained *L. riparium* gametophytes were incubated into the same amount of DMSO used for the 100 μM MCB solution and used as a negative control of MCB. Three samples of *L. riparium* gametophytes for each treatment and control condition were examined.

2.4. Photochemical efficiency

Photosynthetic efficiency was assessed using the widely used and classical indicator F_V/F_m, which indicates for the Photosystem II (PSII) the potential quantum yield of primary photochemistry (Maxwell and Johnson, 2000), and the performance index (PI_{ABS}), a global indicator that resumes the contribution of all parameters on PS I and PSII functionality which was also used to express the overall vitality of the samples. The energy cascade from light absorption by PSII to electron transport involves the absorption of photon flux by antenna pigments (ABS), creating excited chlorophyll. The excitation energy in part is dissipated (DI) as heating and fluorescence emission, and in part is profitably addressed to the reaction centre (RC) as trapping flux (TR); in the RC the excitation is converted into redox energy by reducing the electron acceptor Q_A to Q_A- which is then reoxidised to Q_A leading to the electron transport (ET) and later to CO_2 fixation (Strasser et al., 2004).

PI_{ABS} is given by the equation:

$$PI_{ABS}=RC/ABS\times\varphi P_0/(1-\varphi P_0)\times\psi_0/(1-\psi_0)$$

where φP_0 expresses the probability that an absorbed photon will be trapped by the reaction centre of PSII, it represents the maximum quantum yield of primary photochemistry and roughly corresponds to F_V/F_m, and ψ_0 expresses the probability that a trapped exciton, a quantum of electronic excitation, enters the transport chain and moves an electron further than Q_A.

Measurements were carried out with a Plant Efficiency Analyser (Handy PEA, Hansatech Instruments Ltd, UK) at the temperature of 20 ± 1.3 °C. After dark-adaptation for 30 min, gametophytes were lightened for 1 s with a saturating excitation pulse (3500 μmol s^{-1} m^{-2}) of red light (650 nm) from a LED into the sensor and the fluorescence emission were recorded. Nine measurements were taken for each treatment and the fluorescence data were processed by PEA plus software (Hansatech Instruments, Pentney, King's Lynn, UK).

2.5. Transmission electron microscopy

Gametophyte fixation was carried out with a 3% (v/v) glutaraldehyde solution in a phosphate buffer (pH 7.2–7.4) for 2 h at room temperature. Post-fixation was performed with buffered 1% (w/v) OsO_4 for 1.5 h at room temperature. Dehydration employed ethanol up to propylene oxide and was followed by embedding into Spurr's epoxy medium. Ultrathin (50 nm) sections were collected onto 300 mesh Cu grids

and stained with Uranyl Replacement Stain (Electron Microscopy Science, Hatfield, PA, USA) and lead citrate. A Philips EM 208 S TEM was employed for observation and nine samples for each treatment were examined.

2.6. NADH-GOGAT and NADH-GDH activities determination

NADH-GOGAT and NADH-GDH were extracted by grounding 150 mg of *L. riparium* in a buffer containing 100 mM KH_2PO_4 buffer (pH 7.5), 2 mM EDTA, 2 mM dithiothreitol (DTT) and plant-specific proteases inhibitor cocktail (Sigma P9599, Merck-Sigma-Aldrich, USA). GOGAT and GDH activities were measured by following the oxidation of NADH at 340 nm using a Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, USA) at 25 °C. as described previously (Jallouli et al., 2019).

2.7. Western blotting

Proteins were extracted by grinding samples in liquid nitrogen 300 mg of tissue, then powder was suspended in 600 μl of solution containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol, 15 μM $NADP^+$, 1 μl/30 μg plant-specific proteases inhibitor cocktail (Sigma P9599, Merck-Sigma-Aldrich, USA). Then, proteins were separated by SDS-PAGE and transferred on a nitrocellulose membrane (Ge Healthcare) using the Transblot-turbo (Biorad, CA, Usa) as previously described (Ayadi et al., 2020). Membranes were incubated with primary antibodies for Glutamine synthetase 1 and 2 (GS) and for nitrate reductase (NR) (Agrisera, Vannas, Sweden). After incubating the membrane with horseradish peroxidase (HRP)-linked secondary antibody, cross-reacting polypeptides were identified by enhanced chemioluminescence (ECL) reaction (Ayadi et al., 2020).

2.8. Statistical analysis

Statistical significance of PCn, GSH and photochemical efficiency data was inferred through two-way ANOVA, followed by Tukey's post-hoc test. Data were reported as the mean ± SE. The threshold of statistical significance was set at $p < 0.05$, unless otherwise specified.

3. Results

3.1. Production of thiol-peptides

The production of thiol peptides in response to heavy metals was evaluate by HPLC-ESI-MS-MS analyses to understand whether they were one of the main detoxification mechanisms involved (Fig. 1). The amount of GSH detected was almost constant even in the presence of high concentrations of heavy metals. Differently, the PCn were produced, although at trace levels, in response to heavy metals. Indeed, the PCn levels progressively increased with the increase of heavy metal concentrations. Furthermore, PCn with higher degrees of polymerization were only produced in samples exposed to S2 and S3 with the highest concentrations of heavy metals.

3.2. Confocal laser imaging

In S-unexposed and MCB-unstained samples (Fig. 2.a), autofluorescence from the cell wall (blue) and chloroplasts (red) was visible. After staining with MCB, all the samples showed both MCB signals from both the cytoplasm underneath the cell wall and the vacuoles and chloroplast autofluorescence (Fig. 2. b-e). The S3-treated samples gave a slightly higher blue signal.

3.3. Photochemical efficiency

Chlorophyll fluorescence was used to further evaluate the effects of heavy metal stress on photosynthesis. Control samples (not reported) of *L. riparium* showed no changes in chlorophyll fluorescence over a period of 7 days. While on the contrary (Table 1) both F_V/F_m and PI_{ABS} decreased gradually after exposure to heavy metal stresses in *L. riparium* indicating that the potential quantum yield of primary photochemistry and the overall vitality of the samples are degraded.

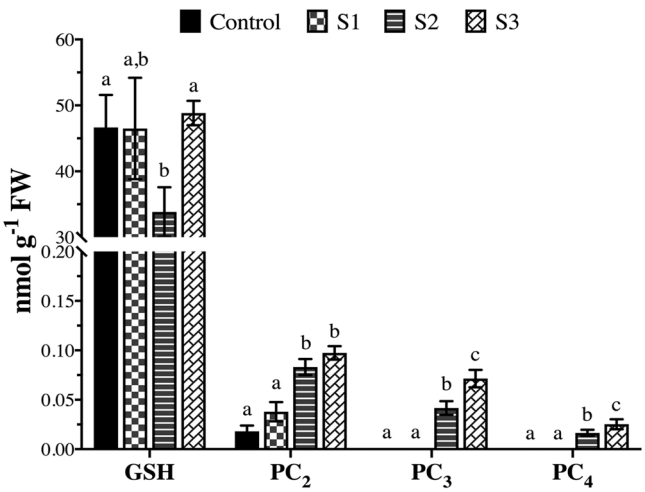

Fig. 1. Content of GSH and PCn in *L. riparium* gametophytes, exposed to Control Solution, S1, S2 and S3 for 7 days. Values are mean ± SE; within each group of thiol peptides, bars not accompanied by the same letter are significantly different at $p < 0.05$ (one-way ANOVA followed by Tukey's multiple comparison post-hoc test).

3.4. Transmission electron microscopy

TEM observations were already shown in Maresca et al. (2018). Chloroplasts from the S2- and S3-treated samples developed ultrastructural changes. S1-treated samples have chloroplasts with a regular appearance (Fig. 3). In the S2-treated specimens, some thylakoids were swollen, whereas in the S3-treated samples chloroplasts were misshaped, thylakoids appeared ill-defined and ill-distinguishable and large lipid droplets developed in the stroma (Fig. 3).

3.5. Nitrogen uptake and metabolism

The effects of heavy metals on nitrate reduction and nitrogen metabolism in different sites were analysed by monitoring the enzymatic activities of GDH and NADH-GOGAT; and protein occurrences of GS1, GS2 and NR. As shown in Figs. 4 and 5, both nitrate reduction and nitrogen metabolism were significantly influenced by different concentrations of heavy metals observed in sites 1, 2 and 3.

GDH represents an abiotic stress responsive gene, playing major roles both by an alternative nitrogen assimilation enzyme both for the re-assimilation of the excess of ammonia released during stress (Zhou et al., 2015; Jallouli et al., 2019). As shown in Fig. 4A, the activity of GDH significantly increased in site 1, 2 and 3 compared with controls. Particularly, the heavy metals induced an increase of GDH of about 4.48, 6.67 and 9.61-fold change by comparing S1, S2 and S3 vs control, respectively.

NADH-GOGAT showed marked and significant increased activities at higher concentrations of heavy metals of site 2 and 3, while a reduced increase was reported at higher heavy metals levels observed in site 1 (Fig. 4B). In details, heavy metals induced an increase of NADH-GOGAT of 2.62, 12 and 14-fold change comparing S1, S2 and S3 vs control, respectively. Immunoblotting analysis showed an increased occurrence of the chloroplastic GS2 (44–45 kDa) in site 2 and 3, while the GS1 (39–40 kDa) showed difference only in S3 (Fig. 5).

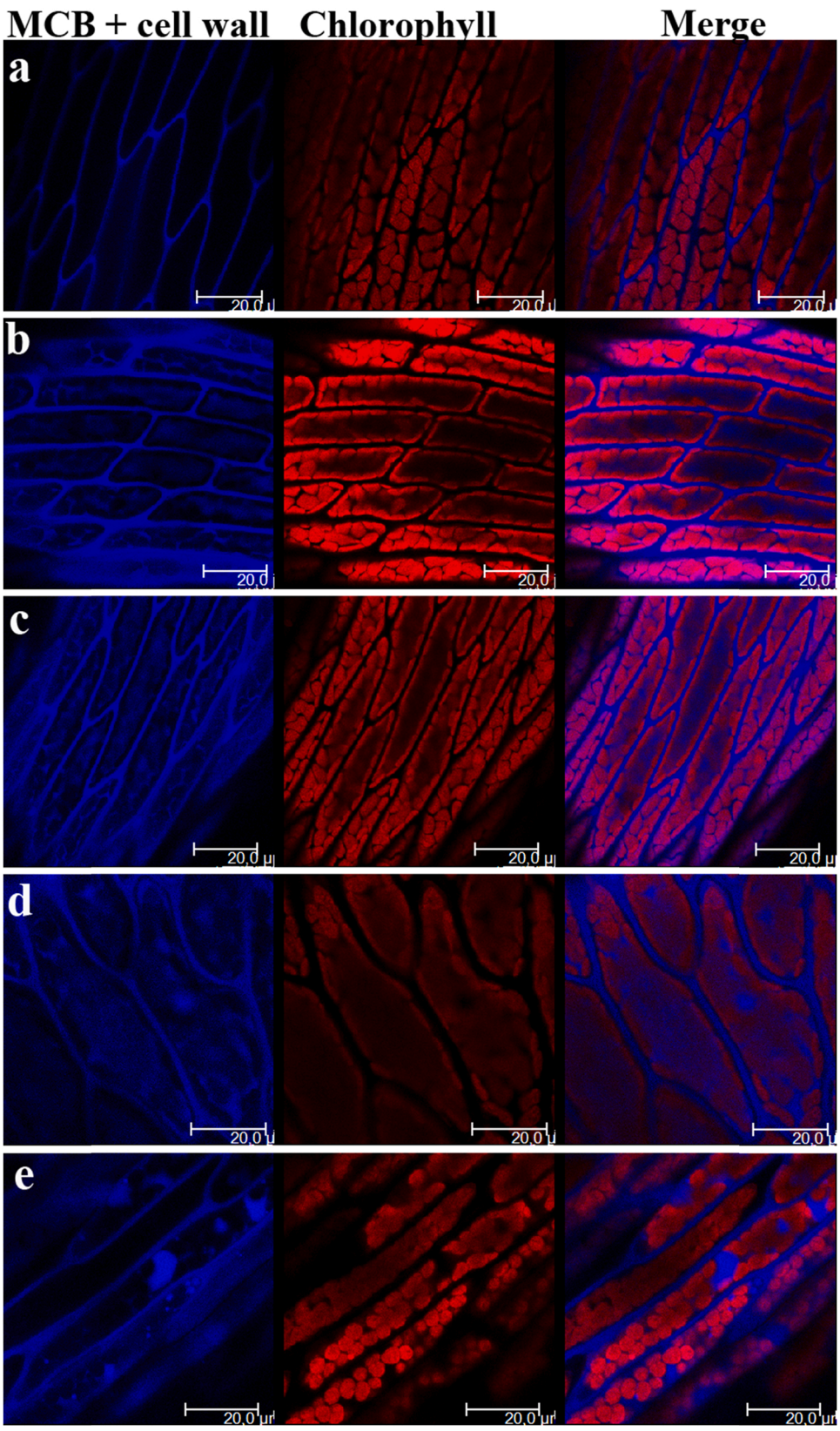

Fig. 2. Confocal laser scanning microscopy (CLSM) micrographs of *L. riparium* phylloids treated without (a, b) and with S1 (c), S2 (d), and S3 (e) solutions for 7 days, unstained (a) and stained with MCB (b-e). The I column shows MCB signal and cell wall autofluorescence, the II reports the chlorophyll autofluorescence and the III is the merge. (a) In the S-unexposed, only DMSO-treated samples, autofluorescence is visible from the cell wall (blue) and chloroplasts (red). (b) In the S-untreated, MCB-stained samples, MCB signal is already visible from the cytoplasm beneath the cell wall and the vacuoles (blue). (c-e) In the S1-S3 treated, MCB-stained samples MCB signal still localises in the cytoplasm underneath the cell wall and the vacuoles, with a slightly higher emission in the S3-treated specimens (e). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NR showed a slight increase in occurrence at lower heavy metals concentrations, while reduced changes were reported among controls and site 2 and 3, respectively.

4. Discussion

The present study gives new insights into the molecular events underlying the metal-tolerance of the aquatic moss *L. riparium* exposed to environmental heavy metal concentrations. Heavy metals play a central

Table 1
F_V/F_m: indicator of photosynthetic efficiency; PI: performance index; Different letters in each column indicate statistically significant ($p < 0.05$) differences between treatments.

	F_V/F_m	PI_{ABS}
S1	0.811 ± 0.052	0.792 ± 0.024
S2	0.731 ± 0.020	0.286 ± 0.012
S3	0.612 ± 0.014	0.148 ± 0.017

role in the physiology of plants, providing essential in some cases, micronutrients for plant growth, as well as being toxic in other cases. An excess of heavy metals may induce toxic effects on plant growth, development, and reproduction (Basile et al., 2012a; Esposito et al., 2018; Lentini et al., 2018). In this study we simulated environmental pollution conditions commonly found in natural conditions (Regi Lagni, Maresca et al., 2018) to further investigate about the effects of naturally occurred conditions induced by anthropogenic influence. Different parameters were evaluated to give a comprehensive overview of heavy metals effects on moss physiology. We found that exposure to heavy metals induced severe effects in *L. riparium* plants causing reduced photosynthesis, phytochelatin and GSH content and on nitrogen metabolism. It is well known that nitrogen and the metabolism of heavy metals are strictly related (Landi and Esposito, 2017; Singh and Prasad, 2017; Lentini et al., 2018; Yang et al., 2020). The heavy metal mix (S1, S2 and S3 conditions) induced significant activation of nitrogen metabolism in L. *riparium*. Our results clearly demonstrated an activation of GS2, GOGAT and GDH upon all stress conditions tested. Generally, N is connected to abiotic stress response, by competing for reductants necessary for the antioxidant response, particularly in the presence of metals and metalloids (Giansoldati et al., 2012; Lentini et al., 2018; Ben Azaiez et al., 2020). At the same time, N and divalent cations shared similar transporters (Mao et al., 2014). An excess of N could increase the uptake of Fe, Zn, Cu, Ca, Hg and other cations, depending on plant species (Yang et al., 2020). In contrast, several higher crops (e.g. maize, pea, bean, and rice) showed lower activity of GS upon uptake of heavy metals (Lee et al., 2013; Saini et al., 2021). The overexpression of GS in rice has been reported as an effective strategy to counteract the effects of Cd exposure (Lee et al., 2013). Our results showed a natural predisposition of L. *riparium* to increase the GS2 protein occurrence. Similarly, *Pisum sativum* and *Populus* plants subjected to chromium (Cr) or Cd exposure reported an increased GDH and GS enzymatic activity, respectively (Gangwar et al., 2011; Zhang et al., 2014). Particularly, GDH plays critical roles upon abiotic stresses functioning both as an alternative N assimilation path and detoxifying the excess NH_4 released during perturbing conditions (Zhou et al., 2015; Jallouli et al., 2019; Ben Azaiez et al., 2020). Upon heavy metal exposure, metals were chelated in roots and moved to the shoots using GSH and PCn (Yang et al., 2020). An adequate N uptake is necessary to maintain this chelation process regulating the GSH and non-protein thiols content and regulating the expression of PC synthase and GSH synthase genes expression

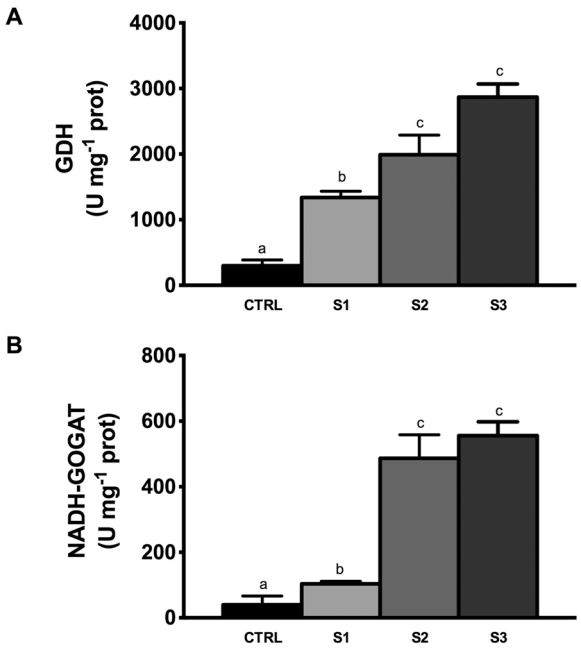

Fig. 4. GDH (A) and NADH-GOGAT (B) activities in leaf of *L. riparium* exposed for 7 days to control (black bars), S1 (light grey bars), S2 (medium grey bars) and S3 solutions (dark grey bars). Letters indicate significant differences between control, S1, S2 and S3.

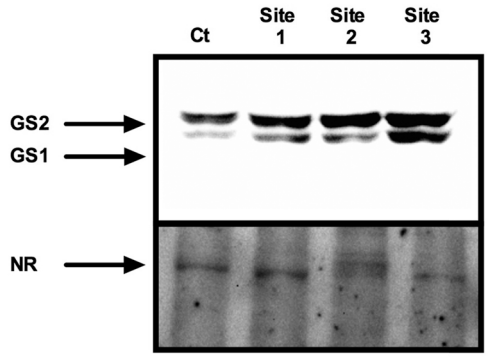

Fig. 5. Immunoblotting of leaf extract of *L. riparium* exposed for 7 days to control, site 1, site 2 and site 3 solutions using antibodies raised against Glutamine synthetase 1 and 2 (GS1 and GS2 – 50 – 40 kDa) and against nitrate reductase (NR – 104 kDa).

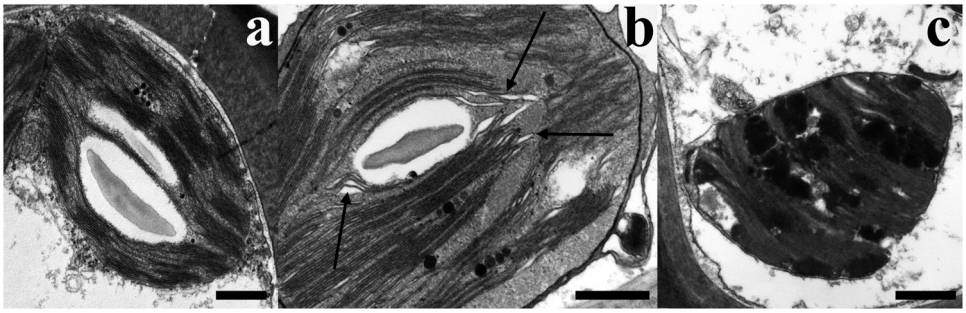

Fig. 3. TEM micrographs of chloroplasts from samples exposed to S1 (a), S2 (b), and S3 (c). **a.** A typical chloroplast with grana and intergrana thylakoids and starch grains. A few plastoglobules are visible in the stroma. **b.** In the chloroplast swollen thylakoids are well evident (arrows). **c.** A misshaped chloroplast where thylakoid membranes are ill-defined, grana and intergrana thylakoids are not well distinguishable and large lipid droplets are present. **Scale bars 500 nm**.

(Finkemeier et al., 2003; Innocenti et al., 2007; Yang et al., 2020).

The synthesis of GSH and PCn were observed also via biochemical approach confirming that *L. riparium* counteracts heavy metal stress with a detoxification system employing thiol peptide compounds (Bellini et al., 2020). Specifically, PCn synthesis was promptly induced by the exposure of heavy metals, but PCn with higher polymerization degree (PC_3 and PC_4) were synthesized only at higher concentrations. Moreover, relatively high GSH levels were detected both in the controls and in treated samples. In fact, GSH levels do not seem to be influenced by heavy metal treatment, probably due to an efficient and prompt synthesis of GSH that compensates for its use for PCn synthesis. The confocal imaging results show that MCB signal is emitted from the cytoplasm underneath the cell walls and the vacuoles of control and exposed samples (S1, S2 and S3), with a slight increase only in the S3-treated specimens. That is consistent with our chemical data, showing presence of GSH and PCn in all the examined samples, with a higher amount of PCn in the S3-treated specimens. The localization of labelled thiols in both the cytoplasm and vacuoles also agrees with a previous study on *L. riparium* with two different Cd concentrations: staining was reported from the same cell compartments with differential distributions related to the Cd concentrations and staining times (Bellini et al., 2020).

The ultrastructural appearance of the samples reflects the cellular stress induced by the increase in the concentration of heavy metals (Maresca et al., 2018). Heavy metal-induced damage to membranes or depletion of energy inside the cell can lead to swelling and/or shrinkage of cell compartments because of loss of selective permeability, a well-known function typical of biological membranes (Bellini et al., 2021). The ultrastructure alteration of chloroplasts was related to heavy metal concentrations in field and in vitro experiments on bryophytes (Esposito et al., 2018; Maresca et al., 2020b; Basile et al., 2009, 2012a, 2012b, 2013). In addition, chloroplasts appeared regular in S1-treated samples, while in S2- and S3-treated samples they developed changes. The ultrastructural damages of chloroplasts can also be explained with the overproduction of ROS, which eventually leads to lipid peroxidation of cell membranes (Farmer and Mueller, 2013), injury to thylakoids (Blokhina et al., 2003) and development of a senescent appearance (Prochazkova et al., 2001). S3-treated chloroplasts showed worse damage to cell membranes with ill-defined thylakoids and accumulation of lipid droplets probably from damaged membranes (Dalla Vecchia et al., 2005; Zhang et al., 2007). The severe alterations observed in S3-treated agree with our data on photosynthetic efficiency, which progressively decreases from S1- to S3-treated specimens. Our results support both the potential quantum yield of primary photochemistry and the overall vitality of *L. riparium* samples exposed to Cu, Zn, Cd and Pb concentrations, in a simulation lab experiment of polluted conditions, are degraded. These results strengthen the suggestion that this moss responding to heavy metal pollution could be suitable for biomonitoring activities in field conditions. Nevertheless, considering that our results concern conditions where two or more metal contaminants co-exist in the natural environment, it is still difficult to judge the contribution of each metal to the biological response.

Despite these findings, several obstacles exist; for example, in the study of Basile et al. (2012b) on different mosses, intracellular concentrations of heavy metals that act as micronutrients, such as Cu and Zn, remained rather constant regardless of their extracellular concentrations, while the accumulation of the elements with no metabolic function, such as Pb and Cd increased, with increasing metal supply in the environment. Moreover, in the study of Rau et al. (2007), comparing equimolar metal concentrations, Zn and Pb treatment in the range of 25–100 μM caused in not measurable influence of the metals on chlorophyll fluorescence in F. antipyretica moss, while Cu and Cd concentration of 100 μM, significantly decreased fluorescence. Thus, the relationship between extracellular and intracellular metal concentrations is metal dependent and the chlorophyll fluorescence measurements show a metal-specific influence of the potential quantum yield of primary photochemistry. Nevertheless, considering that a) most of the previous research focuses on stress of certain, separate heavy metals (Rau et al., 2007), b) two or more metal contaminants usually co-exist in a natural environment, c) mosses often suffer many heavy metals at the same time in the real natural conditions, and d) other environmental variables may influence the biological response, we provide a promising methodology, which needs further calibration and standardization to show the relationship between pollution and the biological responses of potential plant bioremediators, and to assess the contribution of each metal to different biological responses.

In this paper, the moss *L. riparium* is confirmed as an excellent bioindicator of heavy metal pollution as it responds with metabolic variations consistent with the extent of stress. While all the biological responses considered can be used as indicators of a general stress situation, the study of variations in the presence of phytochelatins is particularly interesting as it can be considered a specific indicator of heavy metal stress. Finally, it should be emphasized that presently the induction of phytochelatins in response to metals in the Bryophyta has been reported only in *L. cruciata*, exposed both in vitro (Degola et al., 2014) and to environmental pollution (Maresca et al., 2020b) but data were lacking on how mosses respond. Therefore, this represents the first work to our knowledge that demonstrates the induction of phytochelatins in relation to environmentally relevant concentrations of heavy metals in mosses.

CRediT authorship contribution statement

Adriana Basile, **Sergio Esposito, Stergios Pirintsos** and **Luigi Sanità di Toppi**: Conceptualization, Supervision, Writing – original draft preparation; **Viviana Maresca**, **Erika Bellini** and **Simone Landi**: Methodology, Formal analysis, Validation; **Piergiorgio Cianciullo**, **Giorgia Capasso, Federica Carraturo** and **Sergio Sorbo**: Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

A special thanks to Karl Joseph Duffy for the careful critical and linguistic revision.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113078.

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