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# Functional and structural biomarkers to monitor heavy metal pollution of one of the most contaminated freshwater sites in Southern Europe

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ABSTRACT

This study evaluated the biological effects of highly polluted freshwater environment (Regi Lagni channels, S Italy) on the aquatic moss *Leptodictyum riparium*, exposed in bags at three sites representative of different environmental conditions and characterized by different heavy metal burdens. Bioaccumulation, ultrastructural alterations, Reactive Oxygen Species (ROS) production, antioxidant enzymes activity and DNA damage were assessed. To better evaluate the biological response of the moss species to heavy metals, the same biological parameters were assessed also in *L. riparium* samples cultured *in vitro* using metal mixtures at the same concentrations as measured at the 3 field exposure sites. Heavy metals were accumulated into the moss tissues causing severe ultra-structural damages at higher concentration case studies, and the ROS production as well as the activity of the enzyme followed a concentration-dependent increase. However, the DNA damage trend suggested a threshold effect that changed between field and *in vitro* experiment. The enrichment factor suggests that the concentration in the most polluted site is close to the upper limit of *L. riparium* to accumulate metals. Overall, combining measures of the morpho-functional traits at different level contribute to improving the knowledge about the tolerance of *L. riparium* to heavy metal stress, suggesting that this moss could be suitable for biomonitoring activity in field conditions.

## 1. Introduction

The Domizio-Flegreo Littoral (Campania Region, Southern Italy) and the nearby inner countryside, known as Agro Aversano, both including the Regi Lagni basin, have been declared as a National Concern Site (NCS) by the Italian Government, because of its huge contamination potential. The Regi Lagni channels are the product of a drainage and canalization work of the ancient Clanius River, acted by the Bourbons in the early 1600s. Since then, the areas surrounding the river have no longer been plagued by flooding, which previously affected the nearby territory. The Regi Lagni consists of a network of straight channels that, collecting meteoric, spring and also waste waters, carry them from the plain north of Naples to the Tyrrhenian Sea, covering a length of about 56 km (Di Martino, 2014). Nowadays the Regi Lagni channels are in a completely careless condition and are affected by severe contamination caused by heavy urbanization and industrialization (mainly chemical industry) as well as intensive agriculture and buffalo farms (Di Martino, 2014; Grezzi et al., 2011; Bove et al., 2011). In addition, their catchment area also includes the notorious "land of fires" and the "triangle of death", sadly known for the illegal waste dumping and the soot fallout from their uncontrolled burning causing harmful contamination of the groundwater and soil. This heavy pollution has been causing since a long time a strong impact on the health of the local population, with a significant increase in cerebrum-vascular diseases, lymphoma and cancers (Senior and Mazza, 2004). The district of Acerra (Naples, Southern Italy), one of the vertices of the "Italian triangle of death", emerged as plagued by severe air pollution caused by toxic metals, as shown by biomonitoring studies using mosses and

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lichens (Sorbo et al., 2008; Basile et al., 2008, 2009, 2012a, 2012b).

Depending on their chemical form and bioavailability, it is well-known that toxic metals affect plants, inducing different kinds and extents of damage, impairing anatomy, ultrastructure and molecules and adversely affecting their physiology and biochemistry (Nagajyoti et al., 2010). Ultrastructure damage in plants is a marker closely related to metal pollution (Barceló and Poschenrieder, 2004; Basile et al., 2015). Previous works demonstrated that *Bryophyta* developed ultrastructural changes in relation to metal pollution extent (Basile et al., 2011, 2012a, 2012b, 2013; Esposito et al., 2012). Furthermore, toxic metals lead to overproduction of Reactive Oxygen Species (ROS) in plants. This can trigger redox-sensitive pathways that lead to different alterations, such as protein carbonylation, DNA damage, activation of kinase cascades and transcription factors, which ultimately affect cellular essential metabolic activities and viability (Demidchik, 2015; Shahid et al., 2014). In particular, studies have shown that DNA damage measured in plants using the Comet assay is a good tool for the assessment of genotoxicity of polluted environment (Gichner et al., 2009; Al Khateeb, 2018; Nanda and Agrawal, 2018), detecting DNA single strand breaks and alkali-labile damage in individual cells (Gedik et al., 1992; Singh et al., 1988). Thus using the parameters obtained from Comet assay would allow implementing the intervention strategies to prevent or reduce the deleterious health effects in the sentinel species, as well as in humans. Indeed, assessment of environmental risk requires indicator organisms that quantitatively and qualitatively score the damage and have the capacity to counteract the oxidative pressure caused by heavy metals. To do this, plants have an efficient system of enzymatic and non-enzymatic antioxidants that work in synergy for scavenging the ROS in different compartments inside plant cells (Das and Roychoudhury, 2014). Among these enzymes, superoxide dismutase (SOD) is the first line of defence against ROS, dismutating $O^{2-}$ oxygen molecule and $H_2O_2$. Another enzyme is catalase (CAT) that breaks $H_2O_2$ to water and oxygen while peroxidase (POX) scavenges $H_2O_2$ in chloroplast and cytosol of plant cells. Glutathione S-transferase (GST) belongs to the family of detoxifying enzymes able to catalyse reactions of binding xenobiotics with GSH. GST plays an important role concerning the neutralization of lipid hydroperoxides generated by heavy metals exposure (Kaaya et al., 1999). Frame the changes in the intracellular redox state though these indicators (Inupakutika et al., 2016; Nath et al., 2016) could help to screen which species are able to accumulate and tolerate a large amount of metals, thus being suitable for biomonitoring and phytoremediation studies.

In previous studies *Leptodictyum riparium* (Hedw.) Warnst, an aquatic moss, showed a higher bioconcentration factor when exposed *in vitro* to Cu, Zn and Pb compared to two higher plants, *Lemna minor* and *Elodea canadensis*, which are commonly used in bioindication and phytoremediation projects (Basile et al., 2012a, 2012b).

The aim of this study is to examine the effects that heavy metals can have on functional traits of the already proven *in vitro* bioaccumulator, *L. riparium* (Whitton et al., 1981; Basile et al., 2011, 2012a, 2012b; Esposito et al., 2012). Moreover, the combination of experiments in the field and *in vitro* can allow evaluating if *L. riparium* could be a suitable bioindicator and could be used for phytoremediation in highly contaminated sites.

## 2. Materials and methods

### 2.1. Plant material

Samples of *L. riparium* were collected from a tap water-filled basin in the Botanical Gardens of the University of Naples “Federico II,” Italy. These samples were used for both the field and *in vitro* experiments. The elemental analysis of initial mosses were performed and the results were reported in the Supplemetary materials (SM 1).

### 2.2. Field experiment

After collection, homogeneous samples of *L. riparium* (ca. 2 g fw), were washed with deionized water and placed into > 49 $mm^2$ – meshed nylon bags, as recommended by Kelly et al. (1987). Six bags were exposed for one week during July 2015 at a water depth of 25 cm in the Regi Lagni channels. The following three sites, characterized by different environmental conditions, were chosen for the moss bag experiment: Avella (Site 1, $S_1$) [40°57'48.5"N 14°35'36.9"E], Acerra (Site 2, $S_2$) 40°56'00.5"N 14°22'56.3"E] and Castel Volturno (Site 3, $S_3$) [40°59'01.8"N 13°58'10.3"E]. The selected sites represent three idealized territorial units: Avella ($S_1$), the control site, is upstream of pollution sources; Acerra ($S_2$) and Castel Volturno ($S_3$) are representative of the two most polluted areas of the area: the "triangle of death" and the "land of fires", respectively. The samples from the six bags exposed in each site, were pulled together the analysis described hereafter were carried out on three subsamples. At each site, three water samples were collected on the day of exposure and the day of retrieval of moss samples for subsequent heavy metal analysis. Physical and chemical properties of the water in the three sites were provided by the national environmental agency are reported in the Supplementary materials (SM 2).

### 2.3. In vitro experiment

The samples collected at the Botanical Gardens, washed with deionized water, were cultured in Petri dishes (10 cm diameter), 20 specimens per dish, using sterile modified Mohr medium, pH 7.5, (Esposito et al., 2012) and in the same medium with the addition of the metal salts.

The cultures were maintained for 7 days in a climatic room and the environmental parameters were set according to the environmental conditions registered in the field. In particular: air temperature was maintained at 20 ± 1.5 °C, and 13 ± 1.3 °C, mean ± SD, during day and night, respectively; relative humidity was 70 ± 4% mean ± SD, 16 h light (Photosynthetic Active Radiation 400 µmol $m^2$ $s^{-1}$)/8 h dark photoperiod. These environmental parameters were chosen according to the period of the year, to obtain similar conditions between field and *in vitro* experiments. The samples were treated with heavy metals (Cd, Cu, Pb, Zn) adding to the medium the metals as soluble salts: $CdCl_2$, $CuSO_4$, $Pb(CH_3COO)_2$, and $ZnCl_2$ with the relative anions as K salts in control solutions. The heavy metals concentration administered to the *in vitro* cultured samples were the same as found in the three field sites, hereafter named as:

- $C_1$, for *in vitro* exposure using $S_1$ metals concentration;
- $C_2$, for *in vitro* exposure using $S_2$ metals concentration;
- $C_3$, for *in vitro* exposure using $S_3$ metals concentration.

The *in vitro* cultures were performed in triplicate and repeated three times. At each time, the moss exposed to the same concentration of heavy metals were pulled together and the analysis described hereafter were carried out on three subsamples.

### 2.4. Analytical determination of metal in water samples and in moss

Heavy metals were determinate in both water samples (from field experiment) and moss (field and *in vitro* experiment). The water samples collected in the field experimental sites were filtered through Whatman paper (no. 42) and analyzed by ICP-MS (Perkin-Elmer Sciex 6100) for the concentration of selected heavy metals: Cd, Cu, Pb, Zn. Analytical quality was checked by analysing the Standard Reference Material SRM 1463d ‘river water’. The precision of analysis was estimated by the coefficient of variation of 3 replicates and was found to be < 10% for all elements.

After both the field and *in vitro* experiments, apical leaflets (2 cm),

were collected and then dried to constant weight at 40 °C and then frozen in liquid nitrogen, pulverized and homogenized with a ceramic mortar and pestle.

About 300 mg of moss powder was mineralized with a mixture of 6 mL of 70% $HNO_3$, 0.2 mL of 60% HF and 1 mL of 30% $H_2O_2$ (ultrapure reagent grade). Digestion of samples was carried out in a microwave digestion system (Milestone Ethos 900) for a total time of 30 min. Concentrations of selected toxic metals (Cd, Cu, Pb, Zn), expressed on a dry weight basis, were determined by ICP-MS (Perkin-Elmer Sciex 6100). Analytical quality was checked by analysing the Certified Reference Material BCR 61 "aquatic moss" (*Platyhypnidium riparioides, Hedw.*) with a recovery percentage of 84%. The Precision of analysis was estimated by the coefficient of variation of 3 replicates and was found to be < 10% for all elements. For both experiments, Enrichment Factor (EF) was calculated as the ratio between of the metal in the plant (mg $g^{-1}$) to the metal in the water (μg $L^{-1}$) (Ahmad et al., 2014).

### 2.5. Ultrastructural observations

Subapical leaflets, collected about 5 mm below the apex, were observed under TEM microscopy. Specimens were fixed in 3% glutaraldehyde in phosphate buffer solution (pH 7.2–7.4) for 2 h at room temperature and post-fixed with buffered 1% $OsO_4$ for 1.5 h at room temperature, dehydrated with ethanol up to propylene oxide and embedded in Spurr's epoxy medium (Basile et al., 2001). Ultra-thin (40 nm thick) sections were put on 300-mesh copper grids, then stained with Uranyl Replacement Stain UAR (Electron Microscopy Science) and lead citrate and observed under a Philips EM 208S TEM (Basile et al., 2001). 54 specimens (18 samples from each site; each set made up of 3 specimens in triplicate collected from different dishes) were observed.

### 2.6. Detection of ROS

A fluorescent technique using 2′,7′-dichlorofluorescin diacetate (H2DCFDA) has been used for quantitative measurement of ROS production. H2DCFDA is de-esterified intracellularly and turns to non-fluorescent 2′,7′-dichlorofluorescin (DCFH). DCFH is then oxidized by ROS to highly fluorescent 2′,7′-dichlorofluorescein (DCF) (LeBel et al., 1990).

Homogenates were transferred to a 96- well plate, incubated with 5 μM H2DCFDA for 30 min at 37 ± 1 °C and analyzed using a with an automatic plate reader. ROS quantity was monitored by fluorescence (excitation wavelength of 350 nm and an emission wavelength of 600 nm).

### 2.7. Response to oxidative stress

One gram of plant material was ground with 1 mL of chilled $NaH_2PO_4/Na_2HPO_4$ buffer (PBS, 50 mM, pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 *g* for 30 min, and the supernatant (enzyme extraction) was collected for protein assay and the determination of SOD, CAT, GST and PEROX activities. Protein concentration was quantified spectrophotometrically at 595 nm according to the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976).

CAT activities were calculated and expressed as a decrease in absorbance at 240 nm due to $H_2O_2$ consumption using a commercial kit (Sigma-Aldrich Co., St Louis, MO, USA) and according to the manufacturer's protocol. Superoxide dismutase (SOD, EC 1.15.1.1) activity was spectrophotometrically determined at 450 nm with a commercial kit (19160, Sigma). The assay utilizes a water-soluble tetrazolium salt that produces a formazan dye after reduction by the superoxide ($\bullet O_2^-$) radical. The rate of reduction with $\bullet O_2^-$ is linearly related to xanthine oxidase activity, which is inhibited by SOD. The result is compared with a standard curve of SOD. One unit of SOD activity is defined as the amount of enzyme that inhibits in 50% of the reduction of $\bullet O_2^-$ per minute at 25 °C and pH 7. Glutathione S-transferase (GST, EC 2.5.1.18) activity was measured using a commercial kit (CS0410, Sigma). The conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST was monitored at 340 nm for 4 min. The reaction mixture contained 4 μl of extract and 196 μl of reaction solution (200 mM GSH and 100 mM CDNB in Dulbecco's buffer at pH 7). The activity was calculated with $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$ (Habig and Jakoby, 1981). A GST unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of the GS-DNB conjugate per minute at 25 °C and pH 7.

Assay kits for Peroxidase Activity (Product no. MAK092) were purchased from Sigma–Aldrich (Castile Hill, NSW, Australia). The fluorometric peroxidase activity assay measures the reaction between hydrogen peroxide ($H_2O_2$) and the Fluorescent Peroxidase chemical probe (Product no. MAK092B), which is catalyzed by the presence of peroxidase in the sample (measured at λex = 535/λem = 585 nm).

### 2.8. Comet assay

The protocol was performed according to Gichner et al. (2004) with some modifications. All operations were conducted under inactinic red light to avoid light-induced damage. After plant treatment, the excised organs (around 150 mg) were placed in a 60-mm petri dish kept on ice and spread with 1.5 mL of cold 400 mM Tris buffer, pH 7.5. Plants have a cell wall that cannot be removed by a lysing step as used in the Comet assay protocol for animal and human cells to remove the cell membrane and to denature proteins. The nuclei for the plant cellular and acellular Comet assay have to be isolated mechanically (Gichner and Plewa, 1998). The material plant was gently sliced using a fresh razor blade. The plate was kept tilted on ice so that the isolated nuclei would collect in the buffer. A nuclear suspension (500 μl) and 1% low melting point (LMP) agarose (500 μl) prepared with PBS were added at 37 °C. The nuclei and the LMP agarose were gently mixed and 80 μl aliquots placed on microscope slides which were pre-coated with 1% normal melting point (NMP) agarose. The drops were then recovered with a coverslip and the slides were placed on ice for 5 min. Then, the coverslips were removed and the slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH > 13). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.72 V/cm (26 V, 300 mA) for 5 min at 4 °C. Finally, the slides were gently washed twice in a neutralization buffer (Tris–HCl 0.4 M, pH 7.5) for 5 min to remove alkali and detergent, and stained with 50 mL/mL DAPI (3 h).

A fluorescence microscope was used to examine the slides, analysing a minimum of 50 randomly selected nuclei from each slide and avoiding overlapping figures. A computerized image-analysis system (CometScore) was employed. Twenty-five nuclei were scored per slide, three slides were evaluated per treatment and each treatment was repeated at least twice. From the repeated experiments, DNA damages, Tail moment and olive moment from each slide were calculated.

### 2.9. Statistical analysis

One-way ANOVA was applied for analysing the differences:

- among sites ($S_1$, $S_2$ and $S_3$) in the field experiment in terms of heavy metals concentration in the water as well as metals concentration, ROS production, functional traits such as anti-oxidant activity and DNA damage;
- among the concentration *in vitro* in terms of metals concentration, ROS production, functional traits such as anti-oxidant activity and DNA damage.

Two-way ANOVA was applied to identify differences in the enrichment factor attributable to metals concentration in the water ($X_1$,

**Table 1**
The concentration of heavy metals ($\mu g\,l^{-1}$) in waters of river measured in the three experimental sites (Avella, $S_1$; Acerra, $S_2$; Castel Volturno, $S_3$). Values are presented as mean ± st. dev; numbers not accompanied by the same letter are significantly different at $P < 0.05$, using the post-hoc Student–Newman–Keuls test. The concentrations found in the water of the three sites in field experiment were used for the *in vitro* experiments ($S_1 = C_1$; $S_2 = C_2$; $S_3 = C_3$).

| | Water | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| **Cu** | 113.83 ± 3.71[a] | 4743.46 ± 24.41[b] | 10,812.52 ± 43.94[c] |
| **Zn** | 262.40 ± 11.51[a] | 4260.64 ± 11.02[b] | 396,728.84 ± 1633.1[c] |
| **Cd** | 27.94 ± 2.60[a] | 1804.90 ± 9.38[b] | 278,743.55 ± 685.84[c] |
| **Pb** | 7.54 ± 1.18[a] | 35.94 ± 4.50[b] | 943.77 ± 22.53[c] |

$X_2$, $X_3$) and to the field and *in vitro* experiments. The assumptions of normality (the Kolmogorov–Smirnov test) and homogeneity of variances (Levene test) were tested and when necessary, the data were log-transformed.

The significance of differences was estimated using the post-hoc Student–Newman–Keuls test at $P < 0.05$. Data were analyzed using the software Statistica, version 7.0 (StatSoft, Tulsa OK, USA).

## 3. Results

### 3.1. Heavy metals in water samples

The concentration of heavy metals in water samples (Table 1) varied greatly according to the sampling site, with the control site of Avella ($S_1$), showed the lowest concentrations, whereas the site of Castel Volturno ($S_3$) showed the highest concentration for all heavy metals investigated. Concentration at $S_1$ was within the Italian legal limit established for Pb ($10\,\mu g\,l^{-1}$), but at $S_2$ and $S_3$ values were markedly above to these thresholds. At site $S_3$ concentrations of Cd and Zn were exceptionally high. The concentrations detected in the three sites were used in the water for the *in vitro* experiments ($S_1 = C_1$; $S_2 = C_2$; $S_3 = C_3$).

### 3.2. Concentration and bioaccumulation factor of heavy metals

The concentration of heavy metals (Table 2) significantly differed between the three sites following a general pattern: $S_1 < S_2 < S_3$ for all over the considered heavy metals. Likewise for the *in vitro* experiment the concentration pattern was $C_1 < C_2 < C_3$.

Fig. 1 shows the EF for the considered heavy metals in the two experiments.

In the field experiment, the EF of Cu (Fig. 1a) reached the highest value in $S_2$, at the intermediate concentration of metals, whereas the lower EF occurred in $S_3$, where the highest metals concentration were detected. In the *in vitro* conditions EF decreased when metals concentration increased ($C_1 > C_2 \approx C_3$). Looking at the differences between in field and *in vitro* experiment, for the lower and the intermediate metals concentration the *in vitro* experiment had the higher EF values ($C_1 > S_1$; $C_2 > S_2$) whereas the EF of $S_3$ and $C_3$ did not show differences. For both Zn (Fig. 1b) and Cd (Fig. 1c), EF showed the same general pattern: as the metals concentration increase, EF decreased in both field and *in vitro* experiments ($S_1 > S_2 > S_3$; $C_1 > C_2 > C_3$). For each level of metals concentration, the *in vitro* EF was higher relative to the field. In the field experiment the EF of Pb (Fig. 1d) decreased from $S_2$ to $S_1$, whereas the $S_3$ presented an intermediate EF. On the other hand, *in vitro* experiment the EF decreased as metals concentration increased ($C_1 > C_2 > C_3$). Looking at the differences between in field and *in vitro*, we highlighted that in the latter conditions the EF was tendentiously lower in respect to what found in the field experiment, even if $S_3$ and $C_1$ showed no significant differences.

It is interesting to notice that EF values between in field and *in vitro* are approximately equivalent, except for $C_1$ that for Cu, Zn and Cd reached values that are about twice those found in $S_1$.

### 3.3. Ultrastructure observations

#### 3.3.1. Field experiment

Moss samples in field exposed at site Avella (Fig. 2a–c), those *in vitro* $C_1$ (Fig. 3a–c) and the unexposed specimens collected from the Botanical Gardens showed the same appearance (images not shown). The leaflet cells, surrounded by thick cell walls, contained lenticular chloroplasts beneath the cell wall. The protoplast is occupied in the middle by a large, clear, vacuole. The thylakoid system appeared well developed, arranged as grana and intergrana thylakoids extending along the main axis of the organelle (Fig. 2b–c; Fig. 3b). Starch grains and rare plastoglobules were also visible in the stroma. Mitochondria with electron dense matrix and clear cristae showed a typical appearance (Fig. 2c; Fig. 3c).

In comparison to the previous, samples in field exposed at the $S_1$ and $S_3$ appeared heavily changed, those from site $S_3$ being the most altered. After exposure at $S_1$, the leaflet cells appeared plasmolysed and chloroplasts were well recognizable in the protoplast (Fig. 2d). The chloroplasts were swollen and showed dilated thylakoids (Fig. 2e, f). Nuclei occurred as remnant structures (Fig. 2e). Mitochondria are severely impaired and no clear cristae are still visible (Fig. 2f).

In the samples exposed at site $S_3$, the cells contained few chloroplasts with respect to those exposed at site $S_1$ (Fig. 2g). Those organelles appeared misshaped and the thylakoid system, and its typical arrangement in grana and intergrana membranes, is not yet discernible (Fig. 2h–i).

Site Avella ($S_1$). (a) The leaflet cross section shows thick walled cells with lenticular chloroplasts containing grana and starch grains. (b) A typical cell with a thick wall and chloroplasts featuring grana and intergrana membranes and starch grains. (c) A typical mitochondrion (m) with cristae is located between two chloroplasts.

Site Acerra ($S_2$). (d) The leaflet cross section shows plasmolysed cells with chloroplasts. (e) The plasmolysed cell contains swollen chloroplasts with dilated thylakoids. Remnants of a nucleus (n) are visible. (f) Remnants of two mitochondria (asterisks) next to a chloroplast with dilated thylakoids (arrows).

Site Castel Volturno ($S_3$). (g) The leaflet cross section shows the impaired protoplasts with only a few severely changed chloroplasts. (h) The cell contains severely altered chloroplasts. (i) A heavily modified

**Table 2**
Concentration of metals ($mg\,g^{-1}$) in *L. riparium* exposed in the field ($S_1$, $S_2$, $S_3$) and *in vitro* ($C_1$, $C_2$, $C_3$) experiments. Values are presented as mean ± st. dev; values not accompanied by the same letter are significantly different at $P < 0.05$, using the post-hoc Student–Newman–Keuls test.

| | Field | | | *in vitro* | | |
|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $C_1$ | $C_2$ | $C_3$ |
| **Cu** | 225.87 ± 2.20[a] | 14,802.85 ± 149.7[b] | 18,307.30 ± 67.33[c] | 862.25 ± 3.22[a] | 12,861.44 ± 12.37[b] | 28,198.17 ± 89.79[c] |
| **Zn** | 2476.87 ± 15.35[a] | 7704.61 ± 5.34[b] | 21,347.58 ± 130.6[c] | 9888.55 ± 321.5[a] | 14,456.60 ± 258.58[b] | 125,643.80 ± 3236.5[c] |
| **Cd** | 145.88 ± 2.24[a] | 2724.41 ± 16.18[b] | 13,019.02 ± 163.8[c] | 286.66 ± 9.22[a] | 6353.90 ± 80.18[b] | 109,600.61 ± 1471.98[c] |
| **Pb** | 83.42 ± 1.77[a] | 742.21 ± 16.7[b] | 13,723.70 ± 98.55[c] | 103.72 ± 3.47[a] | 271.87 ± 9.63[b] | 824.11 ± 44.41[c] |

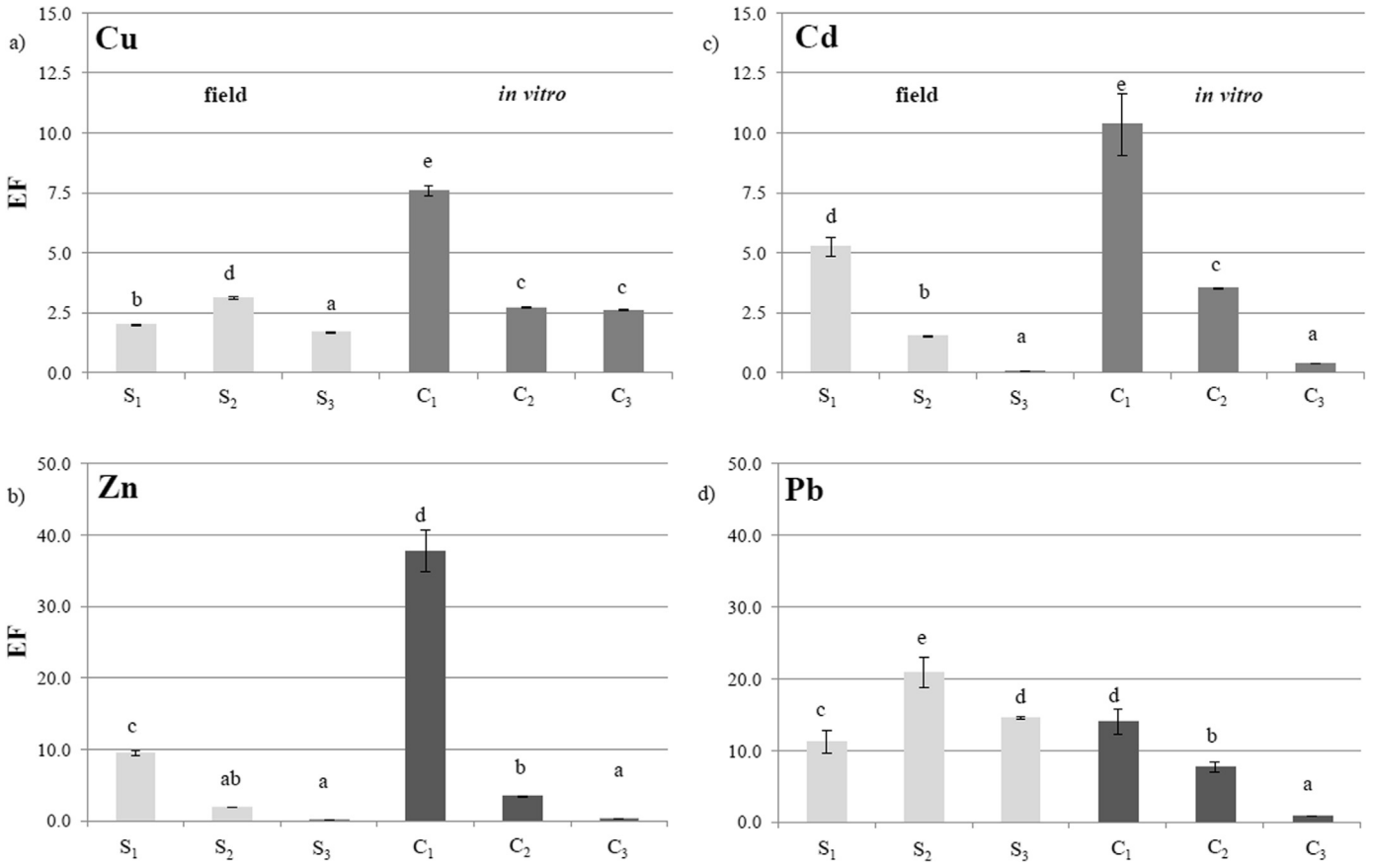


**Fig. 1.** Enrichment Factor (EF) of heavy metals in the field (light grey) and *in vitro* (dark grey) experiments. Values are presented as mean ± st. dev; bars not accompanied by the same letter are significantly different at $P < 0.05$, using the post-hoc Student–Newman–Keuls test.

chloroplast where the typical arrangement of the thylakoid system is no more visible.

Scala bars: 5 μ (a, d, g), 1 μ (b, e, h), 500 nm (i), 300 nm (c, f)

### 3.3.2. *In vitro experiment*

TEM observations of the samples cultured *in vitro* are consistent with the data from the field exposure. Those cultured in the $C_1$ mixture showed features comparable with the site Avella-exposed samples, with a regular appearance (Fig. 3a–c). Differently, those cultured with the $C_2$ and $C_3$ mixtures showed changes similar to the corresponding field exposed ones (Fig. 3d–f, g–i). The appearance of the specimens from the $C_3$ mixture is worse than those from the $C_2$ mixture, consistently with the field data.

### 3.4. *Detection of ROS and activity of antioxidant enzymes*

In the field experiment, the amount of ROS (Table 3) significantly changed among the sites and $S_1$ presented the lower value. The antioxidant activity expressed by SOD was comparable between $S_1$ and $S_2$ although the heavy metals concentration was higher in the latter site. CAT, GST and POX activity increased gradually following heavy metals concentration found in the water of the three sites ($S_1 < S_2 < S_3$, Table 1). Looking at the results acquired during the *in vitro* experiment, the amount of ROS and the antioxidants activity followed the same pattern: $C_1 < C_2 < C_3$, except for CAT where $C_2$ presented higher value relative to $C_3$.

### 3.5. *Comet assay*

In field experiment, the DNA damage detected by comet assay (Table 4, SM 3), significantly increased in $S_3$ site, whereas no differences were detected between $S_1$ and $S_2$.

The results obtained from *in vitro* experiment showed that the % DNA damage, Tail and olive Moment are lower in $C_1$ concentration (equivalent to the heavy metals concentration detected in $S_1$ site) increasing in $C_2$ and $C_3$. In particular, no differences were detected between $C_2$ and $C_3$ for the % DNA damage and olive Moment whereas the tail moment presented a gradual increase from $C_1$ to $C_3$.

## 4. Discussion

The goodness of moss *L. riparium* as bioaccumulator of heavy metals, was tested combining field and *in vitro* experiments, verifying its tolerance through several structural and functional indicators. Compared with values reported for aquatic mosses from unpolluted or lightly polluted rivers (Gecheva et al., 2011; Siebert et al., 1996; Cesa et al., 2015), heavy metal concentrations measured in moss bags were already high at the control site $S_1$. Accumulation of toxic metals in the moss was notably different among sites, and the highest concentrations were reached at the most polluted sites, reflecting the metal content found in water samples. However, the bioavailability of metals could be different between experimental conditions as suggested by the EF that is generally higher *in vitro* relative to the field conditions. Moreover, the general pattern of EF was $X_1 > X_2 > X_3$, but in the field experiment this trend is present only for Zn and Cd. For Cu and Pb, EF varied without a fixed trend suggesting that for these two metals the site-specific conditions are the important drivers of bioaccumulation process (Dixit et al., 2015). Given the very high concentration of metals found in the field conditions, we can claim that *L. riparium* can proficiently accumulate the toxic compounds in its tissue (Basile et al., 2015). However, the EF of $S_3$ and $C_3$ were the lowest for all the considered metals, highlighting that the concentration of heavy metals

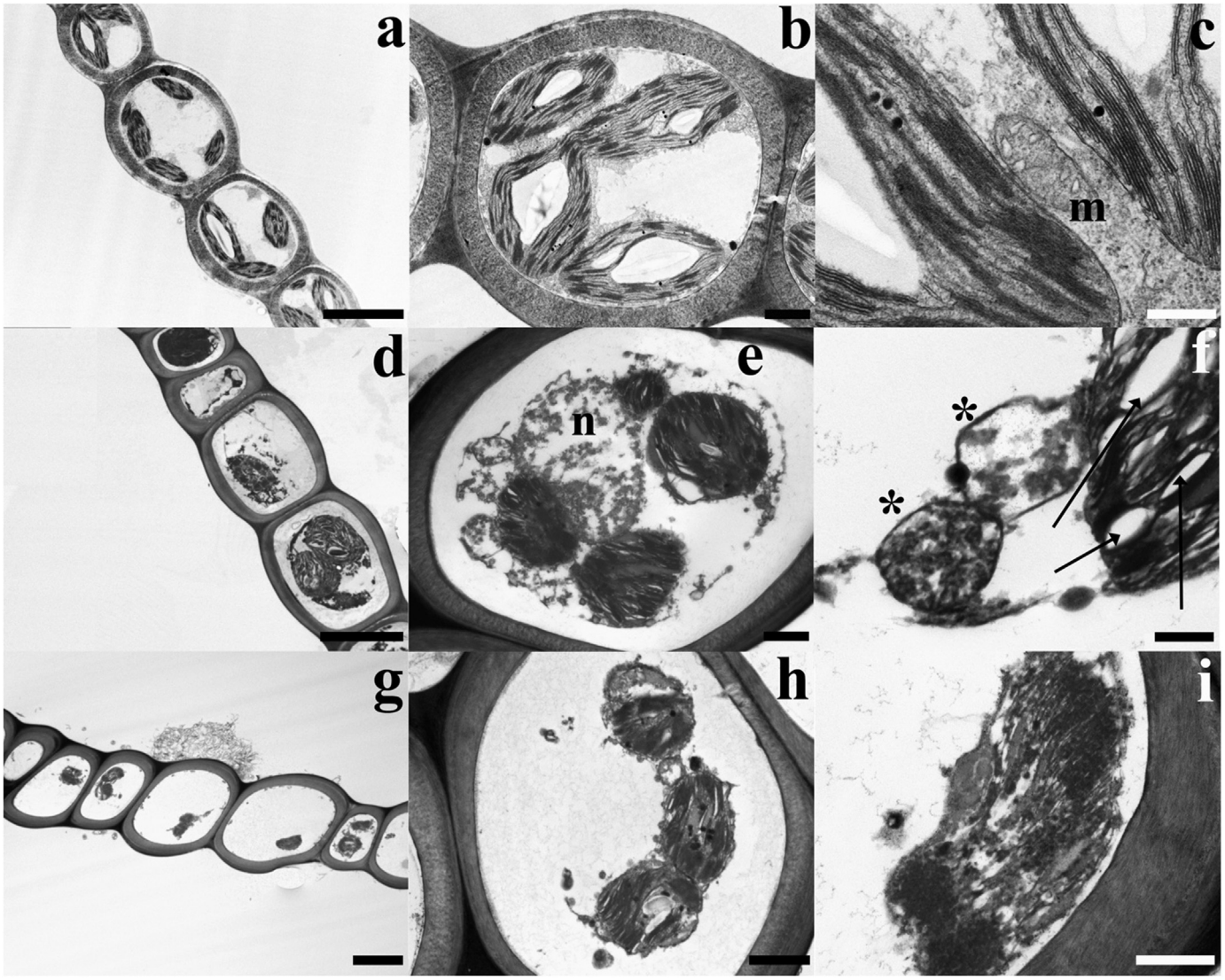


**Fig. 2.** TEM micrographs from leaflets of *L. riparium* specimens in field exposed at the site Avella (a–c), site Acerra (d–f) and site Castel Volturno (g–i).

found in the $S_3$ and used for built the *in vitro* experiment $C_3$, reaches values close to the upper limit of these species for accumulation. This is confirmed by the damages detected at both structural and functional level. Indeed, TEM observations showed that the samples exposed at the control site $S_1$ and the corresponding *in vitro* cultured ones have a regular appearance, with no signs of ultrastructure damage. Compared with these samples, ultrastructural changes were evident at $S_2$ and $S_3$ and at the corresponding *in vitro* cultured ones. These results are consistent with the effects registered in terms of ROS production and comet assays tests related parameters.

The main ultrastructural changes occurred in the chloroplasts and mitochondria. In the samples exposed at the site $S_2$ and cultured in the corresponding metal mixture $C_2$, quite a few thylakoids appeared clearly dilated. Furthermore, also mitochondria appeared severely modified, with loss of cristae, and the shrinkage of the whole protoplast, known as plasmolysis, was also found. In the $S_3$-exposed and mixture $C_3$-cultured samples, the chloroplasts lost the typical arrangement of the thylakoid system, which is even not yet recognizable.

Swelling of organelle and plasmolysis in the plants are morphological hallmarks suggesting an incoming cell death caused by injury, which some Authors call necrosis (Van Doorn et al., 2011). By the way, chloroplasts, and mitochondria, are well-known targets of the toxic effect of heavy metals in plants and even in the moss *L. riparium* itself (Basile et al., 2012a, 2012b; Esposito et al., 2012). The structural damages can be directly ascribed to the overproduction of ROS, which eventually leads to lipid peroxidation in cell membranes (Blokhina et al., 2003; Farmer and Mueller, 2013), injury to thylakoids (Blokhina et al., 2003) and acceleration of cells senescence (Prochazkova et al., 2001). ROS even act as signals leading to cell death (Van Breusegem and Dat, 2006), a process that may also be a trigger in our study by toxic environmental conditions and substances (Schwartzman and Cidlowski, 1993; Van Breusegem and Dat, 2006; Van Doorn et al., 2011) (SM 4). Swelling of organelles or the opposite phenomenon, namelyorganelle or cell shrinkage, like plasmolysis, are caused by the loss of control of selective permeability in the membranes, which in turn is well known to depend either on a direct damage to membrane or secondarily on a cellular energy depletion (Schwartzman and Cidlowski, 1993). Our finding of mitochondria with no cristae, along with plasmolysis and swelling and shrinkage of the organelles, suggests that energy depletion could play a central role in the occurrence of those phenomena. When the control of selective permeability is impaired, as ions move across the membrane along concentration gradients, the accompanying water shifting can cause swelling or shrinkage of organelles, part of them or the whole cell (Schwartzman and Cidlowski, 1993). Beyond structural damages, the evaluation of functional responses it is an important topic in the field of biomarkers,

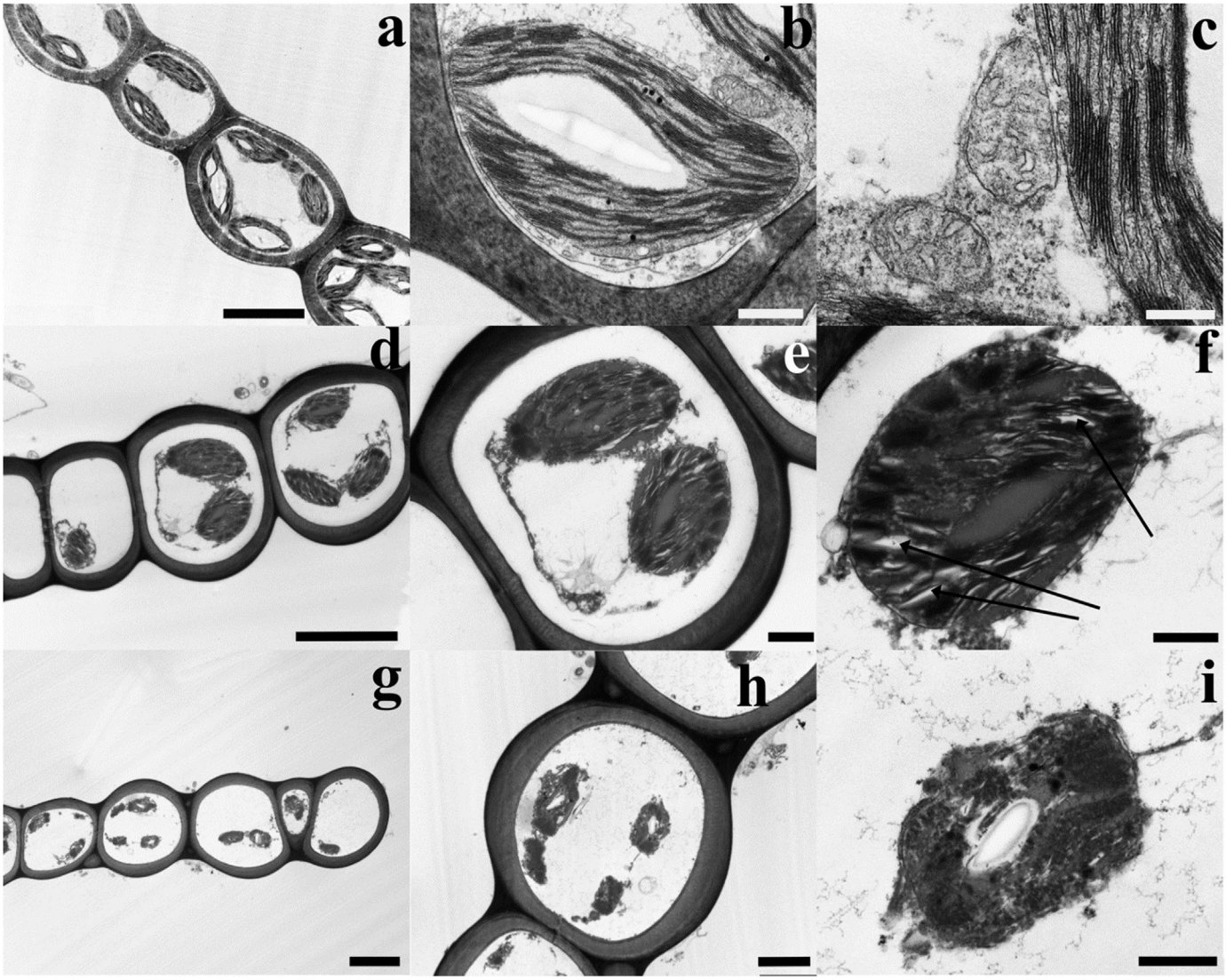


**Fig. 3.** The figure shows TEM micrographs from leaflets of *L. riparium* specimens *in vitro* cultured in the toxic metal mixture at the same concentrations as in the site Avella ($C_1$, a–c), site Acerra ($C_2$, d–f) and site Castel Volturno ($C_3$, g–i). $C_1$. (a) In the leaflet cross section typical thick walled cells show lenticular chloroplasts, with grana and starch grains. (b) The chloroplast contains a well-developed thylakoid system, starch grains and rare plastoglobules. (c) Section of mitochondria with cristae. $C_2$. (d) The leaflet cross section shows plasmolysed cells with chloroplasts. (e) A plasmolysed cell shows altered chloroplasts. (f) A changed chloroplast with dilated thylakoids (arrows). $C_3$. (g) The leaflet cross section shows severely impaired cells containing heavily changed chloroplasts. (h) A plasmolysed cell with severely altered chloroplasts. (i) A miss-shaped chloroplast. The typical arrangement of the thylakoid system is no more recognizable and a central starch grain is still visible. Scala bars: 5 µ (a, d, g), 2 µ (h), 1 µ (e), 500 nm (b, f, i), 300 nm (c).

since the evaluation of environmental quality through the usage of suitable indicator organisms, can help to reach a fast and efficient environmental risk assessment (Huggett, 2018).

Both field studies and laboratory experiments have established that aquatic macrophytes (Maine et al., 2001; Fritioff et al., 2005) and mosses (Bruns et al., 1997; Samecka-Cymerman et al., 2002) can take up water contaminants. This ability and the wide distribution of aquatic bryophytes make them suitable organisms for monitoring metal

**Table 3**
ROS production (fluorescence intensity) and antioxidant responses (SOD, SOD activity inhibition %, CAT, U/mg of protein, GST, umol/ml/min, POX, U/mg of protein) in the field (Avella, $S_1$; Acerra, $S_2$; Castel Volturno, $S_3$) and *in vitro* ($C_1$, $C_2$, $C_3$) experiments. Values are presented as mean ± st. err; numbers not accompanied by the same letter are significantly different at $P < 0.05$. One-way ANOVA was applied for analysing the differences among sites in field experiment, and among the concentration *in vitro*, followed by the post-hoc Student–Newman–Keuls test.

| Field | Sites | ROS | SOD | CAT | GST | POX |
|---|---|---|---|---|---|---|
| | $S_1$ | $60.94 \pm 0.97^a$ | $26.87 \pm 6.73^a$ | $12.94 \pm 0.67^a$ | $1.74 \pm 0.33^a$ | $0.012 \pm 0.001^a$ |
| | $S_2$ | $431.15 \pm 46.80^b$ | $36.06 \pm 4.27^a$ | $35.78 \pm 6.83^b$ | $2.75 \pm 0.04^b$ | $0.029 \pm 0.003^b$ |
| | $S_3$ | $506.72 \pm 69.62^b$ | $59.23 \pm 0.19^b$ | $102.99 \pm 10.44^c$ | $6.55 \pm 1.26^c$ | $0.122 \pm 0.01^c$ |
| ***in vitro*** | **Concentration** | | | | | |
| | $C_1$ | $219.71 \pm 12.84^a$ | $30.65 \pm 2.55^a$ | $0.95 \pm 0.001^a$ | $2.43 \pm 0.04^a$ | $0.03 \pm 0.001^a$ |
| | $C_2$ | $673.26 \pm 49.14^b$ | $47.87 \pm 6.98^b$ | $44.21 \pm 1.80^c$ | $6.24 \pm 0.05^b$ | $0.08 \pm 0.005^b$ |
| | $C_3$ | $928.16 \pm 57.6^c$ | $66.87 \pm 0.27^c$ | $24.00 \pm 2.10^b$ | $7.70 \pm 0.04^c$ | $0.14 \pm 0.01^c$ |

**Table 4**
Effects of heavy metals on comet assay results (DNA damages, Tail moment and olive moment) in the field (Avella, $S_1$; Acerra, $S_2$; Castel Volturno, $S_3$) and *in vitro* ($C_1$; $C_2$; $C_3$) experiments. Values are presented as mean ± st. err; numbers not accompanied by the same letter are significantly different at $P < 0.05$. One-way ANOVA was applied for analysing the differences among sites in field experiment, and among the concentration *in vitro*, followed by the post-hoc Student–Newman–Keuls test.

| Field | Sites | % DNA damage | Tail Mom | olive mom |
|---|---|---|---|---|
| | $S_1$ | $4.32 \pm 0.52^{a}$ | $1.40 \pm 0.19^{a}$ | $1.01 \pm 0.12^{a}$ |
| | $S_2$ | $4.94 \pm 1.82^{a}$ | $1.33 \pm 1.00^{ab}$ | $1.40 \pm 0.20^{a}$ |
| | $S_3$ | $12.95 \pm 0.93^{b}$ | $6.36 \pm 0.27^{b}$ | $3.71 \pm 0.54^{b}$ |
| ***in vitro*** | **Concentration** | | | |
| | $C_1$ | $6.38 \pm 0.87^{a}$ | $1.38 \pm 0.16^{a}$ | $1.72 \pm 0.22^{a}$ |
| | $C_2$ | $10.81 \pm 1.18^{b}$ | $3.35 \pm 0.46^{b}$ | $3.74 \pm 0.44^{b}$ |
| | $C_3$ | $13.02 \pm 1.43^{b}$ | $6.02 \pm 0.70^{c}$ | $4.06 \pm 0.49^{b}$ |

contamination in aquatic environments (Mouvet, 1984).

Different studies have reported total metal contents accumulated by native and transplanted mosses (Siebert et al., 1996; Samecka-Cymerman et al., 2005; Fernandez et al., 2006) or maintained under laboratory conditions (Martins et al., 2004). In some cases, metals were measured both at extracellular and intracellular sites, suggesting possible toxic effects to occur at the cellular level (Vazquez et al., 1999; Fernandez et al., 2006).

However, none of these studies took into account the oxidative pressure exerted by heavy metals on the biomarker as well as their capacity to react. Examine in depth the anti-oxidant capacity of *L. riparium* may help in clarifying the mechanisms involved in heavy-metal toxicity and/or elucidating the cellular basis of moss tolerance of these compounds. Even if some heavy metals (*e.g.* Cu, Zn) are essential micronutrients for plants, all may exert toxic effects, including alterations in photosynthetic and respiration processes or inhibition of plant growth (Vazquez et al., 1987; Kimbrough et al., 1999; Aravind and Prasad, 2005) and being a redox-active metals, they can stimulate the formation of ROS (Prasad, 1999; Wu et al., 2009) leading to multiple toxic effects like lipid peroxidation, protein cleavage or DNA damage (Ünyayar et al., 2006).

To evaluate the oxidative pressure due to heavy metals and their oxidative effects, we measured ROS generation and antioxidant response both in the field and *in vitro* exposed samples. The low level of ROS production confirms that $S_1$ can be considered as a control site relative to $S_2$ and $S_3$, where ROS production is significantly higher. The obtained results underlined that heavy metals could generate ROS through various biochemical processes that lead to the development of a series of defense mechanisms such as SOD, CAT, GST and POX. It is interesting to notice that in the control site ($S_1$) the SOD activity is comparable to that found in concentration *in vitro* $C_1$, despite of the differences in ROS concentration. We hypothesized that in the field a multi-stress condition insists upregulating the first line of defense (Gill and Tuteja, 2010). In the highest heavy metals concentration ($S_3$ and $C_3$ for field and *in vitro* experiment respectively) ROS production was immediately balanced by enzyme activation with an increase of SOD, CAT, GST, and POX. Combing the bioaccumulation data with the biological responses it is interesting to highlight that *L. riparium* showed a clear heavy metal concentration-dependent increase on the response side (anti-oxidant enzymes activity) in both field and *in vitro* experiment, but considering the damage side (DNA damage detected using comet assay) the results suggest a threshold effect that differed between in field and *in vitro* experiment.

Indeed in field experiment between $S_1$ and $S_2$ there were no significant differences for all over the parameters that quantify the DNA damage, despite the presence of heavy metals in the water was markedly higher in $S_2$. On the other side, the damage was evident in $S_3$, confirming the high polluted level of this site.

Looking at *in vitro* experiment, we distinguished a different pattern of damage: the $C_1$, a study case that contains a *concentration* of heavy metals equivalent to the control site in field condition ($S_1$), presented the lower damage level whereas no differences were detected between $C_2$ and $C_3$.

Despite of the concentration of metals are equivalent between field and *in vitro* experiment, the discrepancy found relative to the damage level, could be attributed to the bioavailability of the metals, higher in *in vitro* experiment (Nouri et al., 2011). The combination of field and *in vitro* experiments has highlighted that *L. riparium* can be used as a *bioindicator* for heavy metals and bioaccumulator, giving the responsiveness and its resistance to heavy metals, and other oxidative stresses that can occur in sites highly contaminated by human activities.

Based on the present results, we can conclude that not only higher, but also lower plants (mosses) can be used as an alternative first-tier assay system for the detection of possible genetic damage resulting from polluted waters. In this experimental work, we confirm the extraordinary sensitivity of the method to unveil the damage in *L. riparium* subject to different degrees of toxic metal pollution. Our results corroborate the fact that the occurrence of an alarmingly high level of DNA damage in *L. riparium* exposed in site more pollution can be accounted for the presence of high concentration of heavy metals.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.07.122.

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