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Tissue localization and the physiological effects induced by an environmentally relevant mix of heavy metals in the liverwort *Conocephalum conicum* L. Dum

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ABSTRACT

Tissue accumulation, ultrastructural alterations, oxidative stress, and effects on photosynthesis were assessed in the liverwort *Conocephalum conicom* exposed in vitro to heavy metals (HM) concentrations in three sites of the Savone River, representative of different anthropic impacts. The uptake and accumulation of HM in the thallus of the liverwort was first studied, and the biological effects in relation to the different accumulation sites of HM along thallus, ribs and wings, were then investigated, considering: bioaccumulation (by atomic absorption spectrometry), localization (by X-ray scanning electron microscopy microanalysis), ultrastructural damage of photosynthetic parenchyma (by transmission electron microscopy), oxidative stress (by ROS contents and antioxidant enzymes activities determination), photosynthesis (by chlorophyll fluorescence). The results showed the HM bioaccumulation in *C. conicom* was dependent by their concentrations in the contaminated water. As for spatial localization, HM preferentially accumulated in the nerve of gametophytes respect to the wings. With respect to tissue localization, HM were mainly found in the hyaline and in the photosynthetic parenchyma. Essential metals (Cu and Zn) were accumulated at higher concentrations with respect to non-essential metals (Pb and Cd). At the ultrastructural level, HM caused alterations of the fine structure of the cells, most evident along the nerve, inducing marked alterations of the chloroplast structure and therefore of the photosynthetic capacity. Based on the results of the presented study, *C.conicum* can be used as a marker to indicate heavy metal pollution in water natural resources.

1. Introduction

Environmental pollution is one of the global challenges in the last decades. In particular, water contamination is a major environmental problem. Industrialization, increasing population and urbanization led to worsening of water quality. The main chemical pollutants of water basins are organic matter, nutrients, pharmaceutical and personal care products, poly- and perfluoroalkyl substances, biocides, heavy metals (HM), dyes, radionuclides, plastics (Zamora-Ledezma et al., 2021). Heavy metals are among the most released anthropogenic contaminants; they are not biodegradable and tend to bioaccumulate into primary producers, affecting the entire trophic chain. At a low concentration, some HM are essentials for the optimal physiological functioning of plants (micronutrients: Cu, Zn, Fe, Mn, Mo, Ni, and Co) playing a prominent role in the synthesis of proteins, nucleic acids, photosynthetic pigments, and also taking part in the structural and functional integrity of cell membranes (Arif et al., 2016; Rengel, 1999). Other HM such as Cd, Pb, Hg have unknown physiological role but exert toxic effects also at low concentrations; therefore, their uptake and utilization are tightly controlled by the plant cells (Singh et al., 2016). Plants growing in polluted sites accumulate higher amounts of HM, and these trigger a wide range of physiological and biochemical alterations, in some cases reaching lethal levels. Nowadays, there is an active search for plant hyperaccumulators or markers of heavy metal presence in water and soil

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(Sytar et al., 2016).

Bryophytes, in particular, are able to accumulate and tolerate high concentrations of HM. Many of them survive in contaminated areas and are considered valuable ecological bioindicators across a variety of pollution sources and environments (Maresca et al., 2022a, 2022b). Indeed, bryophytes are pioneer plants that colonized the primitive terrestrial lands (Nickrent et al., 2000) evolving mechanisms to cope with a probably much higher HM load in the environment than today (Degola et al., 2014). The thallose liverwort *Conocephalum conicum* L. Dum (Conocephalaceae), is one of the oldest terrestrial plants (Qiu et al., 1998). It possesses no conducting bundles and no stomata, and the thallus is anchored to the soil with rhizoids (Koselski et al., 2019). The high content of uronic acid, cellulose and protein contents into cell wall, makes *C. conicum* an effective in accumulation of microelement and trace elements (Samecka-Cymerman et al., 1997), which makes this species a useful candidate as a bioindicator of HM pollution.

The aim of this research was to investigate accumulation, tissue localization and possible ultrastructural alteration induced by HM exposure in *C. conicum*. Furthermore, physiological responses, such as photosynthetic performance, pigment changes and oxidative alteration of *C. conicum* were investigated.

2. Material and methods

2.1. Plant material

Samples of *C. conicum* were collected from the mountain site of Savone, identified and deposited in the herbarium of the Botanical Garden of the University of Naples Federico II. Some of these samples were used for in vitro experiments.

2.2. Location and characteristics of the sites

Three different sites of the Savone River were chosen: C1) near the river spring (41° 16' 13.6'' N 14° 02' 19.9'' E); C2) before the entrance of the watercourse in a heavily inhabited area (41° 9' 37.205'' N 14° 1' 59.033''); C3) in correspondence of the town of Mondragone (41° 6' 4.97'' N 13° 53' 33.89'' E).

At each site, three water samples were collected for HM analysis as reported in Maresca et al. (2020).

C. conicum thalli were exposed in vitro for 7 days to the concentrations of heavy metals (Cd, Pb, Zn, Cu) measured in the three different selected sites of the Savone River. The Savone (also called Savone delle ferriere) is an Italian torrent, which originates from some sources in the north-east area of the caldera of the Roccamonfina volcano, between 600 and 650 m above sea level, in the municipality of Roccamonfina. It is about 48 Km long, it has a very sinuous course, enriched by several jumps and waterfalls, and in the section of the Roccamonfina-Foce Garigliano Regional Park it crosses an area rich in woods.

2.3. Gametophyte culture

Field-grown *C. conicum* was gathered in the Savone River site C1. Plants were collected (1–3 cm wide and 5–7 cm long, obtained by cutting the dead basal part). Single gametophytes (1 g) were thoroughly washed with deionized water and then their surfaces were sterilized in 70 % ethanol (2 min) and in 2 % NaClO with a few drops of Triton X-100 (5 min). Subsequently, 20 gametophytes were washed (10 min) with distilled sterile water and put in 5-cm wide Petri dishes with 20 g of fine-granular, washed quartz (Merck). The solution wetted only the lower surface of plants and rhizoids. Gametophytes collected in the upstream site of the Savone river were immediately processed and used as controls. The specimens were cultured with 10 mL of sterile Mohr medium solution in which the same HM concentration measured in Savone River at site C1, C2 and C3 were dissolved. The solutions were replaced every two days. The cultures were kept in a climatic room with a temperature ranging from 13 to 20 °C (night and day temperature), 70 % constant relative humidity, and a photoperiod of 16 h light (40 μmol photons m^{-2} s^{-1}) and 8 h dark. Gametophytes were monitored every two days in order to establish the effect of HM exposure on thallus growth, and browning of tissues. The specimens were maintained in the growth chamber for 7 days. Experiments on gametophyte cultures were conducted in triplicate and repeated three times. The results are the mean ± s.e. of all the observations for each experimental set.

2.4. Analytical determination of HM in water samples and in liverworts

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, and Zn. Analytical quality was checked against 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.

As for *C. conicum* samples, the protocol reported in Maresca et al. (2018) was used. Apical parts (2 cm) were collected, 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 liverwort 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, Elan). Analytical quality was checked by analyzing 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.

2.5. Transmission electron microscopy preparation

Transmission electron microscopy (TEM) observations were performed on specimens prepared as it follows. Collected samples, after careful cleaning, were cut by a sharp blade to pick subapical parts of the thalli, about 5 mm below the apex, from the nerve and the wing areas. After fixation with 2.5 % glutaraldehyde in phosphate buffer solution (pH 7.2–7.4) overnight at 4 °C and post fixation with 1 % osmium tetroxide and 0.8 % KFeCN in the same buffer at room temperature for 1.5 h, specimens were dehydrated with up to 100 % alcohol and propylene oxide and then embedded in Spurr resin. Ultrathin sections, 70 nm thick, collected on 300-mesh copper grids, were stained with Uranyl Acetate Replacement UAR (Electron Microscopy Science) and lead citrate. Observations with a Philips EM 208 S TEM (Basile et al., 2001) focused on the photosynthetic parenchyma that due to the presence of chloroplast is a well-known target of pollution damage as observed in previous studies on liverworts (Basile et al., 2017; Carginale et al., 2004; Maresca et al., 2023, 2022b). Three specimens from each treatment were observed. For each sample, three sections were observed.

2.6. X-ray SEM microanalysis

After HM exposure, plants were thoroughly washed in distilled water for 15 min with several changes of water to eliminate unbound HM and fixed in 2 % glutaraldehyde in phosphate buffer (0.065 M, pH 7.2–7.4) for 90 min at room temperature. The tissue pieces were dehydrated with ethanol, critical-point dried, and mounted on carbon stubs, covered with a 15 nm thick carbon film and observed with a Cambridge 250 Mark 3 scanning electron microscope (SEM). The analysis was performed with an energy-dispersive detection system spectrometer and a Link AN 10,000-analyser computer system (Basile et al., 2001, 1994).

Spectra were collected over 50 s live time with a 0.5 mm diameter

circular probe (spot size); the accelerating voltage was 20 kV and the probe current was 400 mA. The mean count rate was 1000–1500 counts per s and the take-off angle was 35° (Roomans and Shelburne, 1983). Spectra were processed, and quantitative information was obtained by the New ZAFPB/FLS program for deconvolution and background subtraction by least squares fitting of prefiltered spectra (Statham, 1977).

This program was also used for the quantification of data by the continuum method of Hall (Hall, 1979a, 1979b) and (Gupta, 1979). The concentration of the element present is proportional to the ratio of characteristic counts (peak minus background) to the number of counts in the continuum contributed by the specimen. To make a good estimate, counts were collected at 20–40 keV. Quantification was achieved by comparison with standards of known composition. The X-Ray microscope standards, mounted in resin, were supplied by Microanalysis Consultants Ltd, Cambridge. 36 specimens (9 samples from CTRL, 9 samples from C1, 9 samples from C2 and 9 samples from C3 samples in triplicate collected from different dishes) were observed and analyzed by microanalysis.

2.7. Fluorescence parameters determination

The maximal efficiency of photochemistry of photosynthesis in control (not exposed) and HM exposed liverworts was estimated with an IMAGING-PAM M-Series Chlorophyll Fluorometer (Walz, Effeltrich, Germany). The liverworts were acclimated in the dark for 30 min before analysis. After dark adaptation, the maximal quantum efficiency of PSII in the dark (F_v/F_m, where F_v is the variable and F_m is the maximal fluorescence in dark-adapted organisms) was measured.

2.8. Determination of photosynthetic pigments

At the end of the experiment, liverworts were collected, wings and nerves separated by bistoury and immediately frozen in liquid nitrogen and stored at-80 °C. For extraction, frozen samples were ground in liquid nitrogen, homogenized and extracted with N,N-dimethylformamide (1:1 ratio) and transferred into glass tubes. Pigments were extracted in the dark at 4 °C for about 24 h. The absorbance of the samples was measured, using glass cuvettes, at 664, 647 and 470 nm. The Chl-a and Car were calculated according to Inskeep and Bloom (1985) and Wellburn (1994) formula, respectively. The pigment concentrations were calculated according to Salbitani et al. (2021). Chl-b was calculated as the difference between Chl-tot and Chl-a.

2.9. Detection of reactive oxygen species (ROS) and activity of antioxidant enzymes

Wings and nerves separated as described above were collected and used for ROS and activity of antioxidant enzymes determination. One gram (fresh weight) 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 12,000g for 30 min, and the supernatant (protein extract) was collected for protein assay and the determination of ROS levels and SOD, CAT, and GST activities. For each sample, 3 replicates were measured.

ROS levels were assessed using 2',7'-dichlorofluorescin diacetate (H2DCFDA). The extract was incubated with 5 μM H2DCFDA for 30 min at 37 ± 1 °C. ROS quantity was monitored by fluorescence (Ex:350 nm, Em: 600 nm) in 96 well microplates (Falcon™ Fischer Scientific) using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The activity of the antioxidant enzymes catalase (CAT) (Sigma-Aldrich Co., St Louis, MO, USA), superoxide dismutase (SOD) (19160, Sigma, St Louis, MO, USA), and glutathione S-transferases (GST) (CS0410, Sigma St Louis, MO, USA) were measured following the kit instructions. CAT activity (units mg proteins^{-1}) was measured kinetically (for 1 min at 25 °C, 15 s each read) as a drop in the absorbance of H_2O_2 at 240 nm in 2 mL quartz cuvettes. The reaction (1 mL reaction vol) was started adding 500 uL H_2O_2 (10 mM) to 40 μL of protein extract diluted in 460 μL PBS buffer. SOD activity was measured using the xanthine oxidase – WST1. GST activity (μmol min^{-1} mL^{-1}) was assessed through the conjugation of reduced exogenous glutathione with the 1-chloro-2,4-dinitrobenzene (CDNB) The assay was performed in 96 well microplates (Falcon™ Fischer Scientific) measuring the increase in the absorbance at 340 nm of the formed CDNB-glutathione conjugates. The reaction was measured kinetically for 6 min at 25 °C and started adding 20 μL of protein extract buffered with PBS buffer to 180 μL of GSH (40 μM) and CDNB (20 μM).

Protein concentrations were measured with Bradford method using the Bio-rad Bradford reagent (Bio-rad Laboratories, Inc.) in 96 well microplates reading the absorbance at 595 nm.

2.10. Statistical analysis

Data were examined by one-way analysis of variance (ANOVA) and Tukey's test. In all figures, values are presented as mean ± standard error or standard deviation; numbers not accompanied by the same letter are significantly different at $p < 0.05$.

For the student t test significant differences were marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The software used for the analysis was SigmaPlot 14, Systat Software, Inc., USA).

3. Results and discussion

3.1. Heavy metal concentrations in the river

The HM concentration detected in the three sites of the Savone river are shown in Table 1. The concentrations of all HM were within the legal limits at site C1 (Italian Legislative Decree 152/2006). In particular, the most toxic HM such as cadmium (Cd) and lead (Pb) were below detection value. The situation changed in the C2 site, where an increase of all metals was measured, and in C3, a dramatic increase of all HM was observed (Table 1). This increase can be related to the use of the land, essentially agricultural up to site C2. On C3, a widespread fraudulent use of the territory, due to the presence of toxic fires and uncontrolled waste spills towards the terminal area of the river course, around the city of Mondragone, in addition to the obvious effect of extensive urbanization and anthropization, could be the cause of the significant increase of HM. In fact, copper (Cu) can be related to the use of the territory, this time from an agricultural point of view, being copper sulfate ($CuSO_4$) still used as algaecide and fungicide in crops (Weitbrecht et al., 2021). Generally, zinc is released into the environment both naturally (e.g., volcanoes, wind-blown dust, and forest fires) and via anthropogenic activities such as electroplating industry, smelting and refining, mining, biosolids, but the majority of reported contaminations directly affecting people's health was caused by the latter (Li et al., 2018; Tabelin et al., 2018). However, due to the lack of precise data in the land use in the proximity of the Savone river, certain hypotheses cannot be moved toward a punctual characterization of the emission sources.

Table 1
Concentrations of HM found in the Savone River at the sites: site C1 (41° 16′ 13.6″ N 14° 02′ 19.9″ E), site C2 (41° 9′ 37.205″ N 14° 1′ 59.033″) site C3 (41° 6′ 4.97'' N 13° 53′ 33.89'' E) expressed as μg L^{-1}. Details about sites in the Section 2.2 of Materials and Methods.

	Zn	Cu	Cd	Pb
C1	0.4 ± 0.1	1.78 ± 0.4	< 0.01	< 0.01
C2	12.87 ± 2.3	4.35 ± 1.1	0.21 ± 0.1	0.34 ± 0.2
C3	25.3 ± 4.5	7.35 ± 1.6	2.34 ± 1.1	3.41 ± 1.3

3.2. Chlorophyll fluorescence

Fluorescence parameters were measured to assess possible differences in maximal photosynthetic capacity after 7-d exposure to the different experimental conditions (CTRL, C1, C2 and C3). As shown by Fig. 1-A the samples exposed to the highest HM concentration (C3) showed faded colours, which are indicative of photosystem damage, as indicated by the reduced maximal quantum yield of PSII (F_v/F_m, Fig. 1-B). The Imaging-PAM provides us with an in vivo numerical estimate of the F_v/F_m and helps us to understand where the damage is located. The colour of F_v/F_m image was uniform blue in control samples (CTRL). Then, the colour of F_v/F_m image was blue with sporadic green and yellow for HM-treated plants. The colours shifting from blue to yellow indicate great reductions of the maximum efficiency of PSII photochemistry. After seven days of exposure to C3 concentrations, the gametophytes showed a deflection in the F_v/F_m in the nerve area, probably due to the intense transport of water and solutions in the most abundant hyaline parenchyma in this area. PSII is a large pigment-protein complex located into the thylakoid membranes, where it carries out a fundamental role in photosynthetic electron transport (Müh and Zouni, 2020). Therefore, the reduced ratio of F_v/F_m indicates HM toxicity on the photochemistry of photosynthesis.

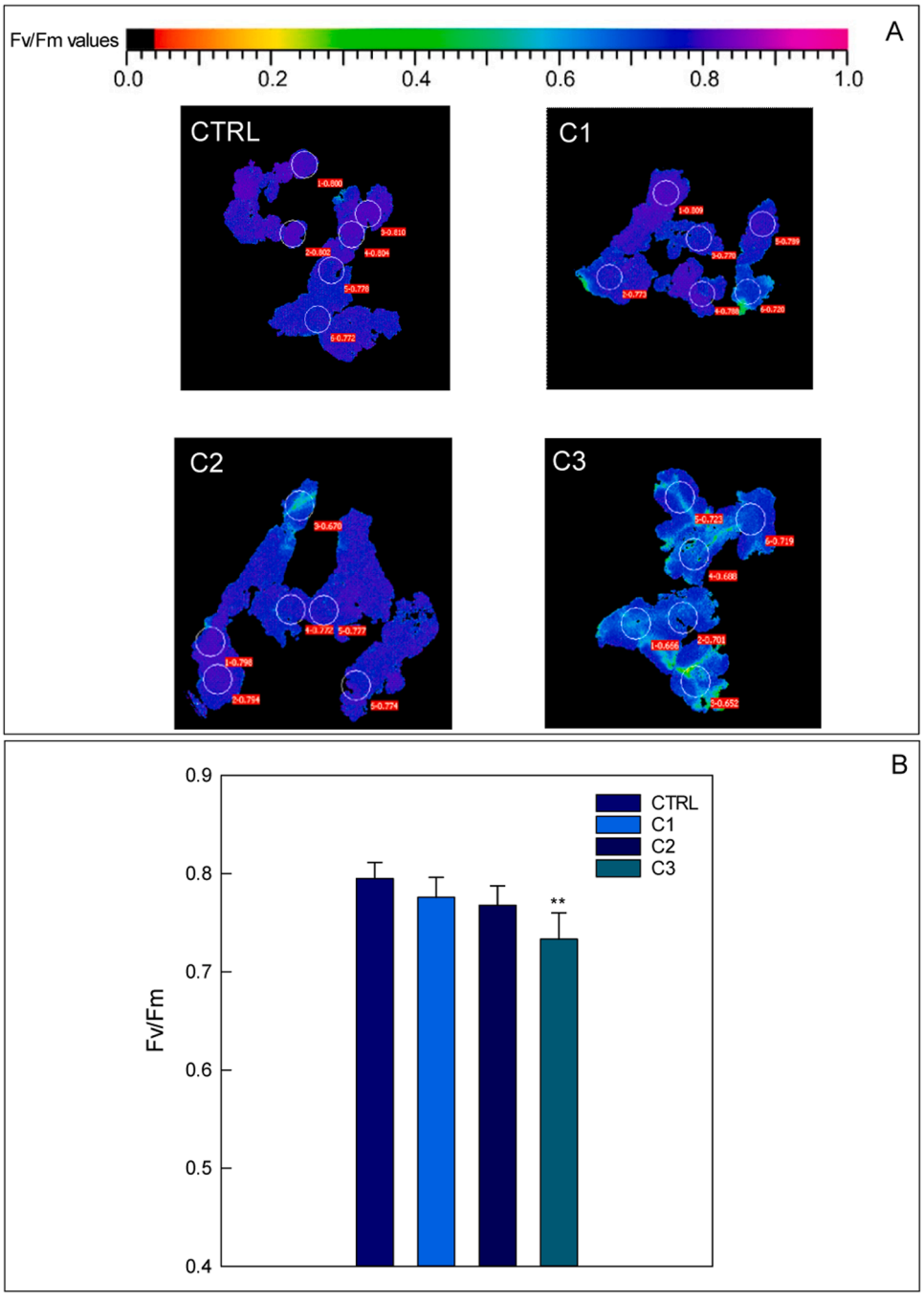

Fig. 1. Maximal quantum efficiency of photosystem II (F_v/F_m). (A) Representative images of F_v/F_m of experimental conditions were obtained by Imaging-PAM. The false-colour scale indicating the F_v/F_m values and ranging from black (0.0) to purple (1.0) is shown. B) Values of F_v/F_m of *C. conicum* after 7 days of treatment (B). Error bars represent SD ($n = 15$). Significant differences respect to CTRL (not exposed sample) were determined by one-way ANOVA with post-hoc Tukey HSD Test ($^{**}p < 0.01$).

3.3. Pigment contents

Pigments such as total chlorophylls and carotenoids were measured in the exposed (C1, C2 and C3) and not exposed (CTRL) samples of *C. conicum* (Fig. 2). A comparison, in pigment contents, between wings and nerves was also made, in the different conditions. A general reduction of pigments was observed in the samples exposed to HM. Already under C1 conditions, a reduction of chlorophylls was observed with respect to controls, but only in the nerves and not in the wings; probably due to lower HM concentration in the surrounding water, the pollutants affected at first nerves and then spread to the wings. The change in photosynthetic pigments content give information on the state of health of the plant. The level of chlorophylls in the cell directly reflects the photosynthetic capacity of plants. In *C. conicum*, the HM exposure reduces the content of both chlorophyll a and b and this reduction (of chlorophylls) could correlate with a damage of photosystems. In particular, the decrease in the content of chlorophylls could be attributed to a reduction of their synthesis due to the inhibition of the reductive steps in the biosynthetic pathways of these photosynthetic pigments (Chandra and Kang, 2016). In fact, the protochlorophyllide

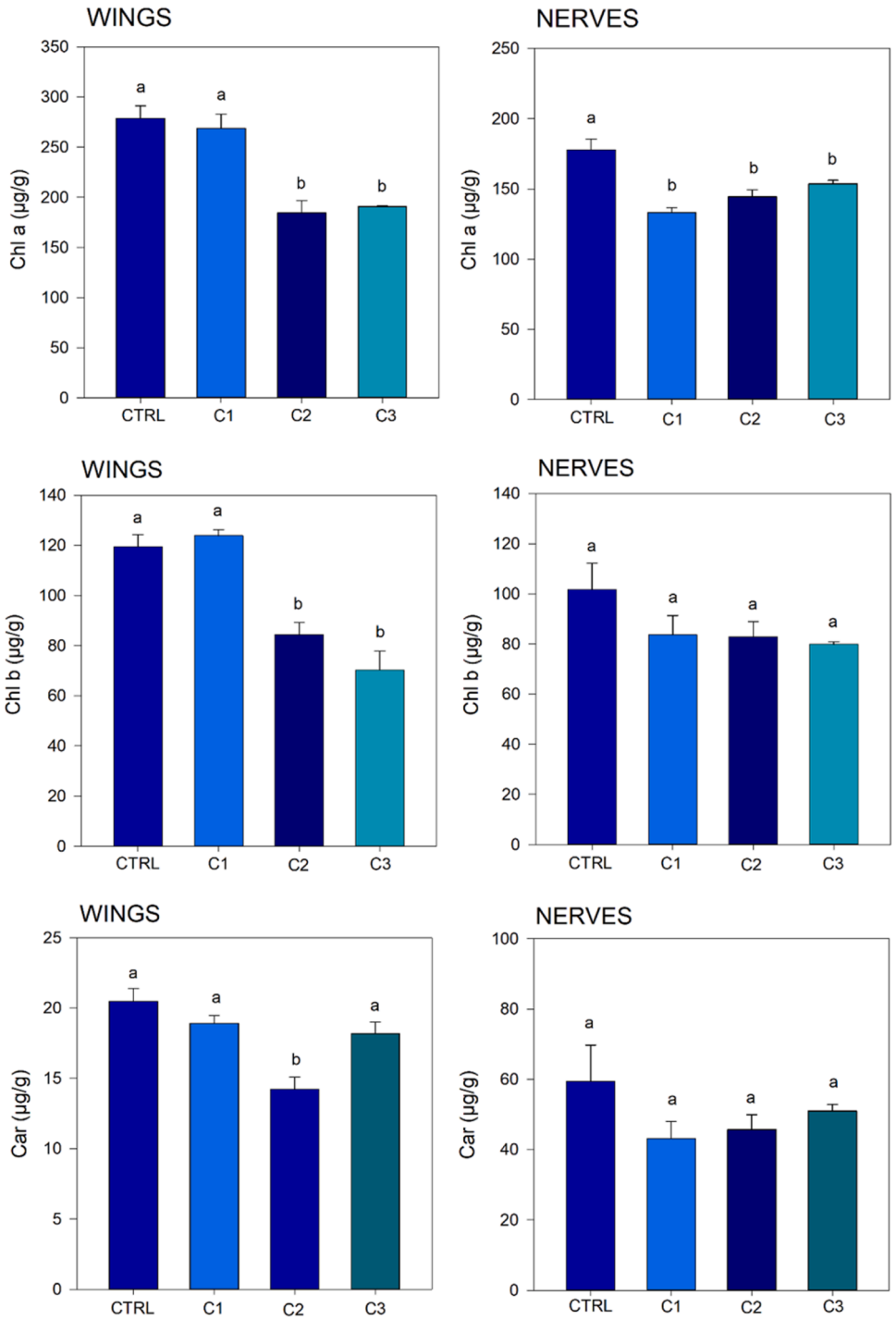

Fig. 2. Pigment contents in control and treated *C. conicum*. The liverworts were exposed for 7 d at different concentrations of heavy metals found in three different sites of Savone river (other details in material and methods). Error bars represent SD ($n = 3$). Significant differences between treated and control samples were determined by one-way ANOVA with post-hoc Tukey's ($p < 0.05$).

reductase, the key enzyme involved in the reduction of protochlorophyll to chlorophyll, is well known to be inhibited by HM. In addition, the photosynthetic membranes are very sensitive to HM and in particular to Cd, which was suggested to firstly affect chlorophyll content, then the photochemical activity of PSII and oxygen-evolving complex, and later the PSI activity (Dobrikova et al., 2021; Wang et al., 2022).

In photosynthesis, carotenoids act as antenna pigments, transmitting the captured light energy to chlorophylls, but they also have the function to scavenge free radicals (Polívka et al., 2004). In fact, carotenoids are known to quench the oxidizing species and triplet state of the chlorophylls, which are involved in the oxidative damage of cellular components (Candan and Tarhan, 2003). Carotenoids in *C. conicum* seem to be less vulnerable to the negative impact of HM as compared to chlorophylls, in fact no important changes were observed in HM exposed plants.

3.4. Detection of ROS and activity of antioxidant enzymes

Antioxidant activity was assessed by measuring ROS levels and antioxidant enzyme activity in different parts of *C. conicum* thalli nerve and wings.

As can be seen from Fig. 3, the C1 samples have ROS levels comparable to the control. In C2 and C3, however, ROS levels increased compared to the control, and in both cases the levels were higher in the nerve than in the wings.

Exposure to HM can cause damage to plant cells directly or indirectly through its increased production of ROS. Plant cells are able to respond to elevated levels of ROS by activating their enzymatic and non-enzymatic antioxidant defence systems.

The main enzymes involved in these defence mechanisms are ROS quenching enzymes such as catalase, superoxide dismutase and glutathione S-transferases. There is growing evidence suggesting stress mechanisms caused by non-Fenton metals (e.g., Pb, Ni, Cd, Cr, etc.) in plant cells may indirectly lead to the production of superoxide radicals, induce lipid peroxidation, increase the activity of some of the key enzymes of antioxidant metabolism and cause severe damages to different cellular organelles and biomolecules (Radotić et al., 2000). This would therefore explain the increased activity of the antioxidant enzymes SOD, CAT and GST in samples C2 and C3 compared to both C1 and the control. The C1 samples showed no significant differences from the controls.

As was the case with ROS levels, enzymes activities were also higher in nerves than in wings. A higher concentration of HM in the nerve than in the wings could explain these differences both in the levels of ROS and of the enzymatic activities in the different parts of *C. conicum* thalli.

3.5. Heavy metal concentrations in C. conicum gametophyte

Zn, Cu, Pb and Cd contents (μg g^{-1}) were measured in the whole thalli of *C. conicum* after a 7-day from HM exposure. As shown in Table 2, the concentrations of the tested HM in the thalli increased with those of the exposure solutions. The absence of HM at C1 (both in water and in *C. conicum* thalli) indicated the excellent preservation of the sampling area. In C2 and C3, *C. conicum* thalli accumulated the highest amounts of HM, with Zn and Cu 32- and 7.6-fold higher than in C1, respectively.

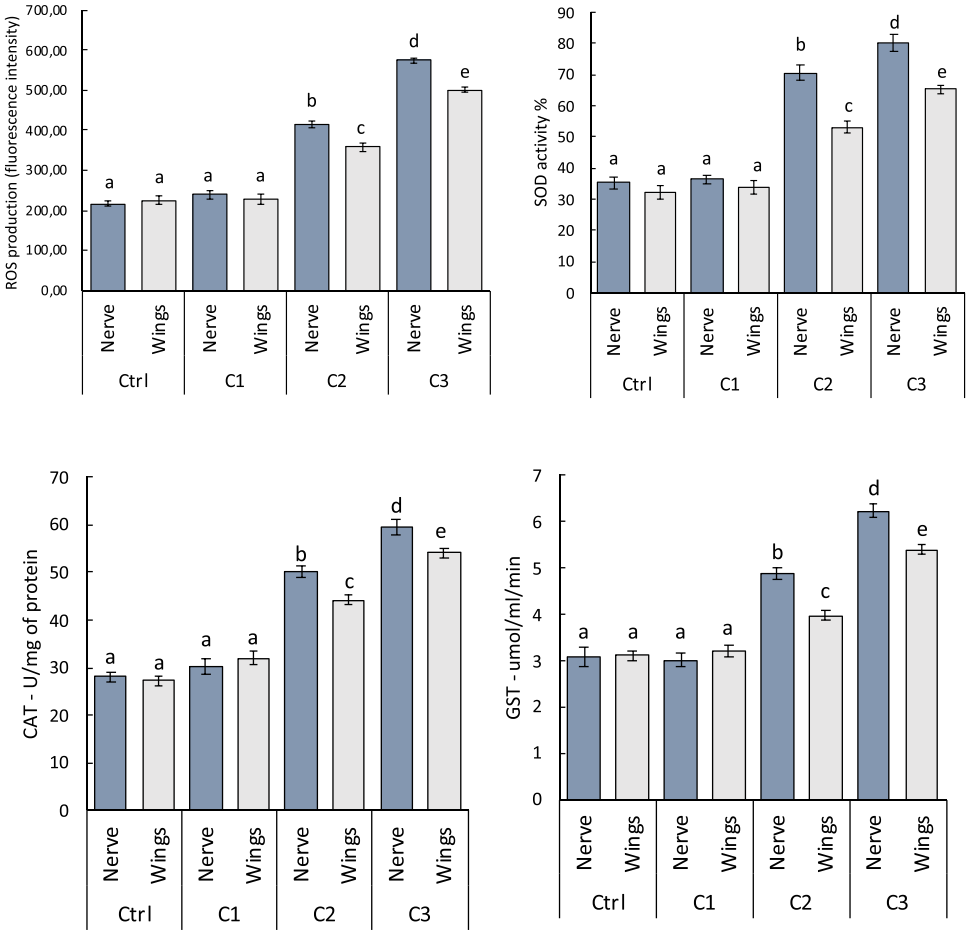

Fig. 3. ROS production and antioxidant/detoxifying enzyme activities SOD, CAT, and GST in the control and in *C. conicum* samples from nerve and wing areas after culturing with C1, C2, and C3 heavy metal mixtures. Bars not accompanied by the same letter (a–e) were significantly different at $p < 0.05$. Data are the mean of three independent experiments ± SE (n = 5).

Table 2
Zn, Cu, Cd, and Pb contents and EC ratios in *C. conicum* gametophytes cultured in vitro (7 days) with C1, C2, and C3 heavy metal concentrations. Concentrations are expressed as mean (μg g^{-1}) ± s.e. EC for Cd and Pb are not determined (n.d.) since their contents in CTRLs are < LOD. Means marked with different letters are statistically significant for one-way ANOVA ($p < 0.05$). The control (CTRL) is the not exposed sample. Comparison between control (not exposed) and exposed samples was expressed as Exposed-to-control ratio (EC) calculated as: $[Concentration\ HM]_{exposed}/[Concentration\ HM]_{control}$.

	Zn	Cu	Cd	Pb
CTRL	1.1 ± 0.1^{a}	4.6 ± 0.1^{a}	< 0.01	< 0.01
C1	1.5 ± 0.1^{a}	5.1 ± 0.9^{a}	< 0.01	< 0.01
EC1	1.36	1.10	n.d.	n.d.
C2	3.2 ± 0.6^{b}	18.4 ± 1.8^{b}	0.8 ± 0.1^{a}	1.3 ± 0.3^{a}
EC2	2.90	4.0	n.d.	n.d.
C3	35.3 ± 2.6^{c}	35.2 ± 3.2^{c}	12 ± 2.1^{b}	6.1 ± 0.7^{b}
EC3	32.10	7.65	n.d.	n.d.

Toxic HM (Cd and Pb) were significantly higher in C3 (12 and 6.1 μg g^{-1}) than the controls (<LOD), C1 (<LOD), and C2 samples (0.8 and 1.3 μg g^{1}). The data are in line with previous studies that tested *C. conicum* for HM accumulation (Maresca et al., 2023, 2020). However, *C. conicum* thallus is composed by layers of differentiated cells along the dorso-ventral axis (Fig. 4). Thus, to investigate the accumulation of the tested HM in the different cell layers, an X-ray SEM microanalysis was conducted.

3.6. Tissue and cellular localization of HM

HM localization and quantification was carried out by X-ray SEM microanalysis of different thallus tissues in gametophytes of *C. conicum* (Fig. 5). HM concentrations (%) were measured along the axis horizontal (wing to nerve to wing) of the thallus. Cd and Pb start being detected in the C2 and C3 with higher concentrations (% metals) in the nerve in respect to the wings (Cd 0.6–2.1 % vs. 1.2–3.3 % and Pb 0.3–2.1 % vs. 0.9–4.6 %).

Whereas, the transverse HM localization showed that the highest % of HM occur in both wings and nerve hyaline parenchyma (2.1 % and 3.3 % respectively). Generally, the HM concentration progressively increased from the upper and lower epidermis to the hyaline parenchyma. The thallus of *C conicum* consists of a cutinized upper epidermis, a thin layer of photosynthetic parenchyma, a layer of hyaline parenchyma, and a lower epidermis provided with rhizoids and ventral scales arranged in opposite rows (Fig. 4). The present data suggest that toxic metals Cd and Pb accumulate mostly in the hyaline parenchyma, where they could be translocated into the vacuoles as a defensive strategy against HM (Basile et al., 2001; Bellini et al., 2020; Degola et al., 2014).

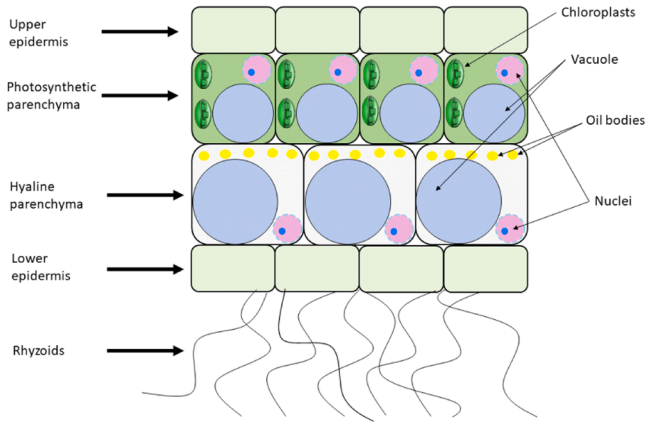

Fig. 4. Scheme illustrating the transversal section of the gametophyte of *C. conicum* as described in detail in the text.

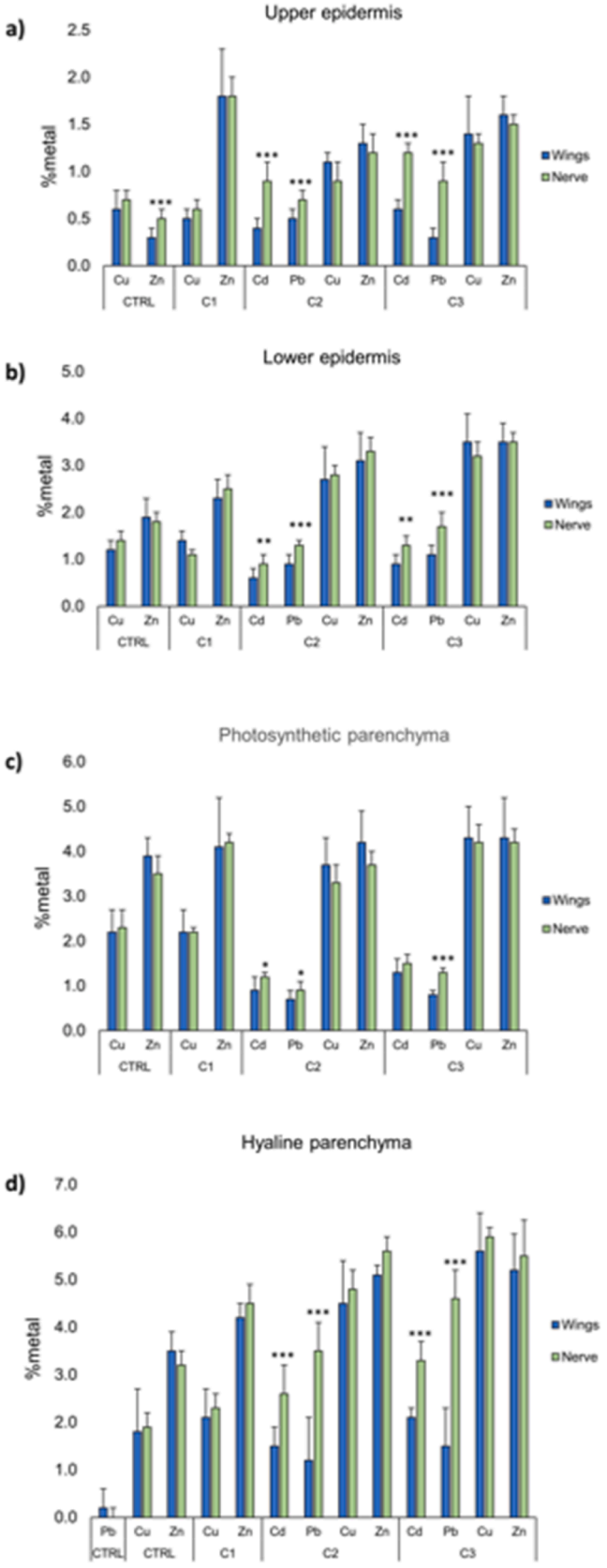

Fig. 5. Mean metals concentrations (%metal) ± st.err. measured through X-ray microanalysis in a) upper epidermis, b) lower epidermis, c)photosynthetic parenchyma, d) hyaline parenchyma of the nerve and the wings of *C. conicum* thalli. Data marked with asterisks are significant for the Student t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The hyaline parenchyma consists of highly vacuolated cells with wall thickening perpendicular to the main axis of cells ("reticulate" cells) and, between walls, a thickening of primary pit fields (Castaldo-Cobianchi and Giordano, 1985). The presence of cell wall thickening could increase the hyaline parenchyma HM adsorption capacity. It has been suggested that the hyaline parenchyma play a role in the symplastic and apoplastic water and solute transport that pass through the rhizoids and the nerve (Castaldo-Cobianchi and Giordano, 1985). In a previous study (Degola et al., 2014), the X-ray TEM microanalysis of the liverworts *Lunularia cruciata* exposed to Cd indicated that this metal, apart from the cell wall, was mostly accumulated into the vacuoles together with increased sulphur content probably due to thiol chelating agents. Present results showed that essential (not toxic) metals (Cu and Zn) were more equally distributed among the tissues than toxic HM (Cd and Pb). These results corroborate the idea of a specific mechanism devoted to the immobilization of toxic HM. Data from imaging PAM (Fig. 1) suggest that after 7 days of exposure the decrease in F_v/F_m is mostly localized in the nerve sector of the C3 exposed thalli. This demonstrates that the concentrations detected through X-ray microanalysis in the nerve area of C3 exposed samples are able to exert a localized alteration of the photosynthetic process.

3.7. TEM observations

TEM observations showed a typical appearance of chloroplasts from both the nerve and wing areas of *C. conicum* thalli cultured with C1 mixture. Under these conditions of exposure to low HM, a well-developed thylakoid system with grana and intergrana thylakoids, starch grains and a few plastoglobules was visible (Fig. 6a, b). Exposure to C2 rendered similar TEM images, with normal chloroplasts on both the nerve and wing areas (Fig. 6c, d). C3 condition was harmful to the nerve area where severe damage was evident, being chloroplasts dramatically swollen, but still showing grana and intergrana thylakoids (Fig. 6e). However, C3 was not so harmful to the chloroplasts from the wing areas, which showed a preserved ultrastructure but an increase of plastoglobules (Fig. 6, f).

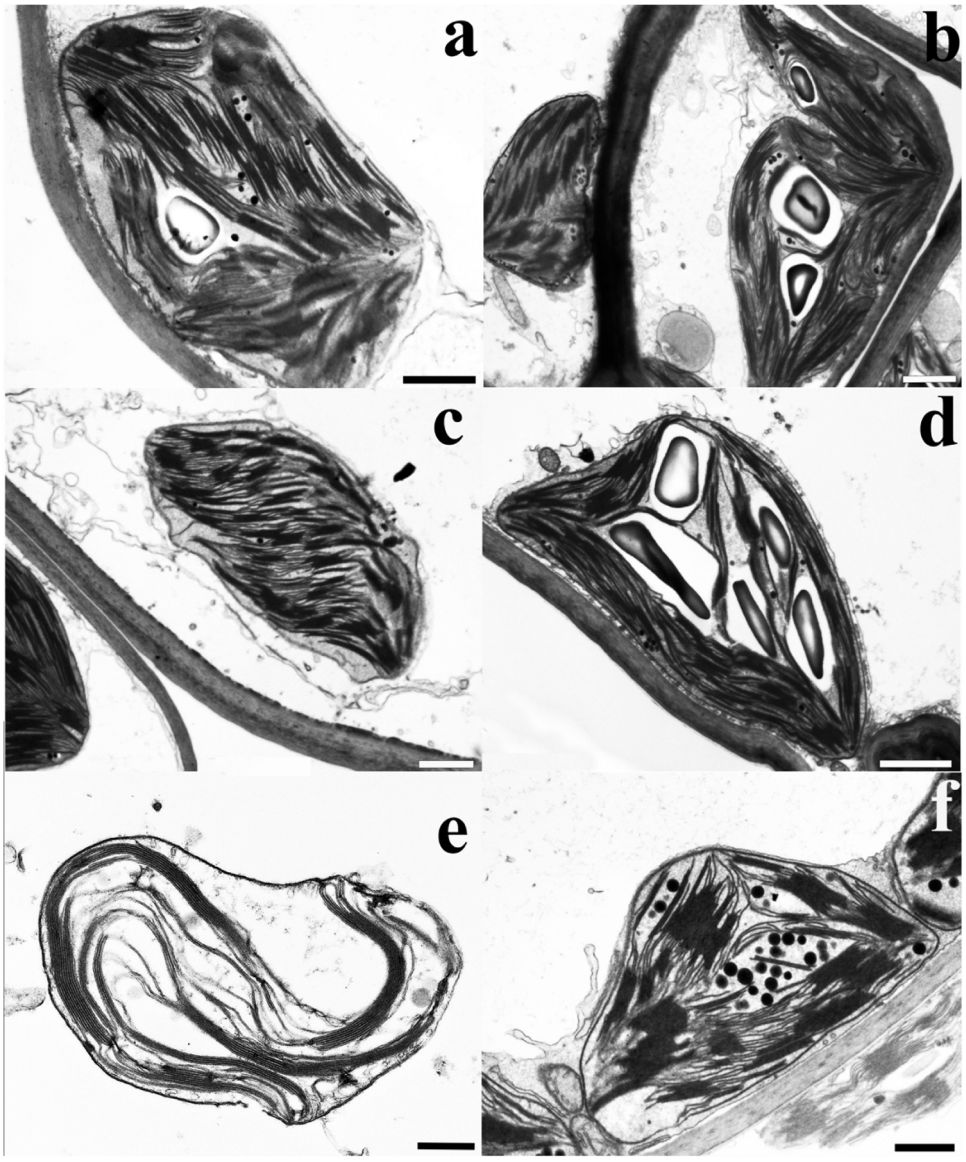

Fig. 6. The figure shows TEM micrographs of photosynthetic parenchyma of *C. conicum* thallus from nerve (first column) and wing (second column) areas after culturing with C1 (a, b), C2 (c, d), and C3 (e, f) heavy metal mixtures. (a) An oblong chloroplast with well-developed grana and intergrana thylakoids, a few small electron dense plastoglobules and a starch grain. (b) Normal chloroplasts with grana and intergrana thylakoids and starch grains. (c, d) The chloroplasts from both nerve and wing areas save a typical appearance: grana and intergrana thylakoids are abundant and starch grains can be visible. (e) A severely swollen chloroplast; grana and intergrana thylakoids are still visible. (f) A chloroplast with an abundant thylakoid system and numerous plastoglobules is taken.

TEM observations of *C. conicum* thalli exposed in vitro to freshwater pollution of Savone river confirmed the findings of Maresca et al. (2022a, 2022b), as they also reported severe damage of the ultrastructure, most of all in chloroplasts, only in samples exposed to the most polluted site in Regi Lagni channels (Italy). Our present experiments indicate that the nerve areas are mostly affected, whereas it is remarkable that the wing areas still maintain most of the typical ultrastructure. A recent in vitro experiment tested *C. conicum* thalli exposed to freshwater pollution at two sites of Sarno river. These TEM observations showed severe alterations of chloroplasts after in vitro culturing with HM concentrations such as those of the downstream site (C3) (Maresca et al., 2023). Previous experiments already demonstrated the sensitivity of plant ultrastructure to freshwater pollution, as shown with the liverwort *Pellia neesiana* (Basile et al., 2017), the moss *Leptodyctyum riparium* (Esposito et al., 2018) and the angiospermophyte *Lemna minor* (Basile et al., 2015). In all cases, local freshwater pollution induced evident ultrastructure changes, downstream conditions being always the most harmful. In our present experiment ultrastructural alterations can also be related to the found increase of HM, most of all the highly toxic Pb and Cd, in the freshwater of the exposition sites.

In the different experiments, including the present one, chloroplasts undergo swelling and show an increase of plastoglobules as observed in the other ultrastructural (Basile et al., 2015; Hakmaoui et al., 2007; Maresca et al., 2023, 2022b). Swelling and shrinkage of the whole cell or its compartments may indicate loss of selective permeability of membranes, which can be caused by a damage to the membrane or may be the effect of an energy depletion (Schwartzman and Cidlowski, 1993). When selective permeability is damaged, ions moving across the membrane according to concentration gradients may change water uptake and distribution, thus causing swelling or shrinkage of membrane compartments (Schwartzman and Cidlowski, 1993). As already shown in a previous work, exposure of *C. conicum* to HM induces ROS (Maresca et al., 2022b), which are harmful to membranes due to lipid peroxidation and to the consequent changes of membrane permeability (Su et al., 2019).

Plastoglobules are a lipoprotein subcompartment in chloroplasts, whose increase is often related to stress and senescence (Bréhélin et al., 2007; Tkalec et al., 2008). Exposure to high concentrations of HM was shown to induce an increase of plastoglobules in different plant species (Minkina et al., 2018). Plastoglobule increases can be related to membrane alterations in plastids, as plastoglobules accumulate lipids, proteins, and pigments released during the reorganization of grana under the effect of stress factors (Titov et al., 2007). However, plastoglobules also actively stock tocopherols (Vidi et al., 2006) that can mitigate photo-oxidation of membrane lipids and photo-inactivation of photosystem II (Havaux et al., 2005). Under oxidative stress, the tocopherols that are stored in plastoglobules can move to thylakoid membranes to scavenge ROS species (Bréhélin et al., 2007). Therefore, we may regard the increase of plastoglobules as an active protective response of plant cells against the stress induced by pollution. Consequently, our data suggest that the nerve area is affected by such a severe damage to metabolism that this active protective response is prevented. That could explain the lacking of that ultrastructure change in the wing area.

4. Conclusion

In this paper the liverworts *C. conicum* was exposed to environmentally relevant concentrations of HM derived from a real case scenario (Savone River). The bioaccumulation and the damage were dependent by HM concentrations in the water. For the first time has been evaluated the tissue localization of HM. *C. conicum* preferentially accumulated HM in the nerve of gametophytes with respect to the wings. HM were mainly found in the hyaline and in the photosynthetic parenchyma. The exposure to HM induces alterations of the fine structure of the cells, most evident along the nerve, inducing marked alterations of the chloroplast structure and therefore of the photosynthetic capacity.

CRediT authorship contribution statement

Giovanna Salbitani: Writing – original draft, Investigation, Data curation, Software. **Piergiorgio Cianciullo:** Writing – original draft, Investigation, Data curation, Software. **Viviana Maresca:** Investigation, Data curation. **Sergio Sorbo:** Investigation. **Marilena Insolvibile:** Data curation, Software. **Francesco Loreto:** Conceptualization, Supervision, Validation, Writing – review & editing. **Alessia Di Fraia:** Writing – original draft. **Adriana Basile:** Conceptualization, Supervision, Validation, Writing – review & editing. **Simona Carfagna:** Conceptualization, Supervision, Validation, Writing – review & editing.

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.

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