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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),