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Effects of heavy metals on ultrastructure and Hsp70 induction in *Lemna minor* L. exposed to water along the Sarno River, Italy

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A B S T R A C T

The effects of freshwater pollution in the highly contaminated river Sarno (Campania, Southern Italy) have been evaluated using bags containing the aquatic plant *Lemna minor* (Lemnacee, Arales), in order to determine morpho-physiological modifications as a response to pollutants.

The exposition of *Lemna* bags for 7 days on three different sites along the river path showed alterations in chloroplasts and vacuoles shape and organization. Moreover, some specimens were exposed *in vitro* at the same heavy metal (HM) concentrations measured in the polluted sites of the river, and compared with data from the bag experiment; to verify the dose and time dependent effects, samples were exposed to HM *in vitro* at concentrations ranging from 10^{-6} to 10^{-4} M up to 7 days.

Transmission electron microscopy (TEM) observations on *in vitro* plants confirmed that ultrastructural alterations affected most of plastids and the shape of different subcellular structures, namely vacuoles; in *in vitro* stressed specimens, Heat Shock Proteins 70 (Hsp70) levels changed, in dependence of changing levels of HM measured in different sites along the river path.

Thus *L. minor* exhibited a possible correlation between the levels of HM pollution and Hsp70 occurrence; interestingly, the data presented showed that copper specifically increased Hsp70 levels at concentrations detected in polluted river waters, whereas cadmium and lead did not; on the other side, the latter represent highly toxic elements when specimens were exposed to higher levels *in vitro*.

The effects of specific elements *in vitro* are compared to those observed in bags exposed along the river path; thus results are examined in order to propose *L. minor* as an organism able to be utilized to monitor heavy metals pollution; the possibility of using Hsp70 s as specific markers of HM pollution is discussed.

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1. Introduction

Environmental pollution is a major issue for human health: namely, poisoning of freshwaters is of special concern, since rivers may transport contaminants very far away from the pollution source, thus hazardously modifying uncorrupted biosystems.

Sarno River (Campania, Southern Italy) is about 25 km long, passes through a large plan comprising highly populated towns from Sarno, where the river springs, to Nocera, Pompei, Scafati and finally flows at Rovigliano in the gulf of Naples; the catchment area of the Sarno is 500 km^2 with a population of about 700,000 inhabitants living along the river basin (Albanese et al., 2013a).

The Sarno is actually supplied by three springs, Foce, Palazzo and Santa Marina, originating three separate torrents joining in Sarno and forming a single main river stream; southwards, at San Marzano – Scafati, the river collects the waters from Alveo Nocerino, an artificial canal, collecting Cavaiola and Solofrana rivers, the latter being concerned by tanneries and therefore by chromium pollution (De Pippo et al., 2006; Albanese et al., 2013a).

The Sarno basin could be divided in two environmental units: the first, corresponding to the hilly and mountain areas is characterized by a low anthropic pressure; the second, the economically developed and densely populated areas of the river plain path, corresponds to the river valley where from moderate to very

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high levels of contamination of waters and sediments are present; the environmental status of these latter areas has been exceptionally compromised by agriculture and industries and by a general disrespect for the ecosystem protection (Albanese et al., 2013a).

The river represents one the most disturbed water ecosystems in Europe: due to the lacking of respect of the laws, occurred deficiencies in the sewerage-purification systems, wastewaters from industries not adequately treated, and crop fields where excess of chemical fertilizers and pesticides are used, have been producing a heavy pollution of the freshwaters (Albanese et al., 2013a; De Pippo et al., 2006), and of the surrounding ecosystem (Adamo et al., 2003). The situation is worsened by the geological nature of the area, where clay and organic matter in the soils and river bed easily adsorb heavy metals, and simplify the transfer of these and other pollutants to the river waters, thus amplifying both the effects of contamination and the environmental risk (De Pippo et al., 2006; Albanese et al., 2013a). This pollution and the disordered urbanization/industrialization of the area have been causing heavy consequences for the public health, with noticeable increase in tumor frequency in these populations (+53% in Hodgkin lymphoma; +20–40% in lung, stomach, bladder and brain cancer – Albanese et al., 2013b). Thus, the catchment of river Sarno was declared “area with high risk of environmental crisis” by the Environment Ministry (Ministry Council's Decree, 1995).

The geological context of the catchment area has played a major part in determining the current ecological conditions and public health problems: while human activity has modified the landscape, the natural order has indirectly contributed to increasing the environmental impact (De Pippo et al., 2006); even if several depollution programs have been structured, so far the pollution of Sarno River is not solved.

It is well known that heavy metals cause severe morphological, ultrastructural and physiological damages in plants: at physiological and biochemical levels, damage can be assessed by observing changes in the occurrence of Heat Shock Proteins 70s (Hsp70s), a fundamental chaperone involved in protein folding, cell homeostasis, and stress response, as recently demonstrated in Bryophyta (Basile et al., 2011, 2013; Esposito et al., 2012). It is well known that Hsp70s are the most abundant chaperons in living cells: different organisms produce distinct and variable isoforms of Hsp70s; moreover a class of these proteins, heat shock cognate Hsc70, has been described as a constitutively expressed group of proteins, playing a role in the correct folding the newly synthetized polypeptides (Hartl, 1996). Hsp70s are generally indicated as the stress inducible forms, and considered to have a variety of functions, including increasing Hsc70 occurrence, recovering partially unfolded proteins, and facilitating the removal of denatured proteins (Hartl, 1996).

The observed increase of Hsp70 levels in response to environmental stress has led to the proposal of their potential use as biomarkers (Lewis et al., 1999; Bierkens, 2000); therefore, Western blotting for Hsp70s has recently been proposed as a method to detect pollutants effects in worms (Nadeau et al., 2001), and Bryophyta (Basile et al., 2012b; 2013).

Most of the species belonging to the Lemnaceae (duckweeds) family are widely used as model hydrophytes in ecotoxicology, due to their wide distribution, fast growth, short life span, and sensitivity to environmental changes (Lemon and Posluszny, 2000; Lemon et al., 2001); these small floating or submerged hydrophytes expand nearly exclusively by recruitment of asexual propagules (Landolt, 1986); *Lemna minor* is used in standard protocols for toxicity tests (ISO 20079, 2001 or OECD 221, 2002).

In a previous study on the free-floating *L. minor* L., (monocotyledonous angiosperm – Arales) we assessed tissue accumulation, intracellular localization, and toxic effects of cadmium (Cd), lead (Pb), zinc (Zn), and copper (Cu) (Basile et al., 2012a): *L. minor* ultrastructural damage was observed following nickel exposition; the damage was mainly detected in chloroplasts shape, inducing a transition from chloroplasts to chloro-amyloplasts (Appenroth et al., 2010).

L. minor has the capability to uptake heavy metals from water *in vitro*, and this caused an increase in the occurrence of Hsp70, as previously demonstrated (Ireland et al., 2004; Tukaj et al., 2011); Hsp70s play a primary role counteracting these toxic effects, protecting proteins from misfolding and proteolytic pathways, thus proposing *L. minor* as a good bioaccumulator for heavy metals, particularly for Cd (Ireland et al., 2004; Basile et al., 2012a). Despite of this evidence, a real and unambiguous correlation between HM stress and Hsp70 occurrence changes must be still acquired for samples exposed in polluted sites.

This study aimed at evaluating biological effects of freshwater pollution in the river Sarno, using nylon bags containing the aquatic duckweed *L. minor*, in order to determine morpho-physiological modifications as a response to pollutants. In addition, the effects of single metals, at the same concentrations measured in the natural sites, were tested *in vitro* to verify the specific effects of HM on the ultrastructural organization and HSPs induction.

The data are discussed in relation to the possibility of using this organism and these methods in biomonitoring, as promising indicators of HM toxicity. These hypotheses were tested in a valuable system, which is both an aquatic angiosperm *L. minor*, whose biological properties are widely used in the research on biomonitoring and phytoremediation projects, and the water environment of the heavily polluted Sarno River.

The aim of this work is to demonstrate that the *in vitro* physiological and morphological response of *L. minor* to heavy metal pollution is comparable to the response of the same species exposed in field to environmental heavy metal pollution. Furthermore we want to demonstrate that both morphological changes and Hsp70 induction can be useful marker of environmental heavy metal pollution.

2. Materials and methods

2.1. Plant material

Field grown plants were collected from the Botanical Gardens of the University of Naples; homogeneous samples were obtained selecting deep green and with no signs of ageing specimens. Plants were thoroughly washed with deionised water and surface sterilized in 70% ethanol (2 min) and in 2% NaClO added with few drops of Triton X-100 (5 min); specimens were then acclimatized for 2 weeks in Mohr medium, modified as follows: KNO_3 100 mg, $CaCl_2$–$4H_2O$ 10 mg, $MgSO_4$ 10 mg, KH_2PO_4 136 mg, $FeSO_4$ 0.4 mg and 1 ml of BBM (Bold Basal Medium) solution (Nichols and Bold, 1965) to 1000 ml distilled water, pH 7.5 in a climatic room described below.

2.2. Bags preparation and exposition

For bag preparation, acclimatized plant material was disposed (2 g fresh weight) in nylon bags (from a 10 cm × 10 cm nylon layer, 1 mm^2 meshed); six bags were located at each site, and exposed floating in the river at the last week of July for 7 days; The average temperature of river waters was between 17.5 and 19 °C.

Three sites were chosen (see Supplemental Fig. 1) for moss bag experiment:

1. Rio Foce (site A) near a spring, representing a highly preserved area;

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