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Mercury induced haemocyte alterations in the terrestrial snail *Cantareus apertus* as novel biomarker

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ABSTRACT

The aim of the present work was to study the response of a suite of cellular and biochemical markers in the terrestrial snail *Cantareus apertus* exposed to mercury in view of future use as sensitive tool suitable for mercury polluted soil monitoring and assessment. Besides standardized biomarkers (metallothionein, acetylcholinesterase, and lysosomal membrane stability) novel cellular biomarkers on haemolymph cells were analyzed, including changes in the spread cells/round cells ratio and haemocyte morphometric alterations.
The animals were exposed for 14 days to *Lactuca sativa* soaked for 1 h in $HgCl_2$ solutions (0.5 e 1 μM). The temporal dynamics of the responses were assessed by measurements at 3, 7 and 14 days.
Following exposure to $HgCl_2$ a significant alteration in the relative frequencies of round cells and spread cells was evident, with a time and dose-dependent increase of the frequencies of round cells with respect to spread cells. These changes were accompanied by cellular morphometric alterations.
Concomitantly, a high correspondence between these cellular responses and metallothionein tissutal concentration, lysosomal membrane stability and inhibition of AChE was evident.
The study highlights the usefulness of the terrestrial snail *C. apertus* as bioindicator organism for mercury pollution biomonitoring and, in particular, the use of haemocyte alterations as a suitable biomarker of pollutant effect to be included in a multibiomarker strategy.

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1. Introduction

Expanding of human activities has produced an increase of soil pollution during the last decades due to the intensive use of fertilizers and biocides in agriculture, industrial activities, urban waste and atmospheric deposition. Some of the most diffusive chemicals occurring in soil are heavy metals. They can enter in the soil from different sources, such as organic and inorganic amendants, pesticides and fertilizers, mining, wastes and sludge residues, and deposition from atmospheric transport following coal and oil burning (Capri and Trevisan, 2002). Unlike to the harmful organic compounds, heavy metals do not decompose and do not disappear from soil even if their release to the environment can be restricted (Chabikovsky et al., 2004). Therefore, the effects of heavy metal contamination on soil organisms and on decomposition processes persist for many years and pose serious problems for living organisms, as well as being difficult to be evaluated. Mercury is one of the highly toxic metals in soil pollution because of its multiple natural and anthropogenic sources, the high volatility of its elemental metallic form and the high atmospheric persistence (UNEP, 2013). Burning of fossil fuels, waste incinerator and mining activity are some of the major source of this metal.

Due to the increasing concern about soil chemical contamination there is an increasing interest in the scientific community and international agencies for soil pollution monitoring and assessment. The traditional approach, based on chemical analysis in order to establish the presence and concentration of specific toxicants, does not provide alone indication about the deleterious effects of contaminants on the biota (Calisi et al., 2011; De Vaufleury and Pihan, 2000). Therefore, the development of new biological tools based on the use of biological indicators has become of great importance for the assessment of the quality of this environmental compartment (Kammenga et al., 2000). The detection of pollutant concentrations in the tissues of bioaccumulator organisms has been identified as an indirect measure of the abundance and availability of metals in the soil. Moreover, the measurement of biochemical, cellular and physiological responses (i.e. biomarkers) developed by bioindicator organisms to soil pollutants are considered early warning signals helpful for gaining insight regarding the exposure and the mechanisms causing observed toxic effects.

Gastropods are among the most successful invertebrates in terrestrial ecosystems (Dallinger et al., 2001). Most terrestrial Gastropods are detritivorous animals, feeding on decaying litter, which exhibits a high capacity for retaining trace elements and organic pollutants on its surface (Dallinger et al., 2001). Therefore, terrestrial gastropods feeding

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on this material can absorb pollutants from their food sources. These organisms exhibit very high capacities for metal accumulation (Dallinger, 1993; Dallinger et al., 2001; De Vaufleury and Pihan, 2000). In doing so they play an important role in directly transferring pollutants to higher trophic levels of terrestrial food chains being prey or hosts for a variety of other animals (Oehlmann and Shulte-Oehlmann, 2002). The main site of accumulation is represented by the digestive gland (Dallinger, 1993), followed by foot sole, mantle, and intestine. The high capacity for metal accumulation and storage is attributed to the induction of metallothioneins which constitute an efficient tool for metal detoxification (Höckner et al., 2011). Previous studies have demonstrated the useful use of snails as bioindicator organisms for heavy metal soil pollution monitoring (Dallinger et al., 2004; Rabitsch, 1996). Snail species such as *Achatina marginata* (Achuba Fidelis, 2008), *Eobania vermiculata* (Bertani et al., 1994), *Helix aspersa* (Courdassier et al., 2001; Beeby and Richmond, 2002), *Helix pomatia* (Dallinger et al., 2005), *Helicella candicans* (Honek, 1993), *Monacha cartusiana* (Ismail et al., 2013) have been validated as quantitative indicators of environmental metal pollution. Although some information is available about metal bioaccumulation and metallothionein induction in terrestrial gastropods, less knowledge is available about other responses to heavy metal exposure in these organisms.

A particularly interesting tissue from a toxicological point of view is represented by the haemolymph, which transports pollutants throughout the exposed organism and its cells are involved in the internal defense system. For this reason any alteration of haemocyte functioning can compromise the health of the entire organism. As previously reported by several authors (Calisi et al., 2008, 2009; Nigro et al., 2006) haemocytes represent one of the first targets of toxic action in invertebrates, so their alterations following pollutant exposure can provide useful information for a more complete understanding of the biological effects associated with exposure to metals. Recently, mollusk haemolymph cells (haemocytes) have received a growing interest for pollutant biomarker development (Calisi et al., 2008; Da Ros and Nesto, 2005). From a morphological point of view two types of cells are recognized in snail haemolymph: round cells and spread cells (Adamowicz and Bolaczek, 2003). Round cells are small, with a high nucleus-cytoplasm ratio. Spread cells are polymorphic cells, with large pseudopodia, polymorphic nucleus and numerous granules in the cytoplasm.

The aim of the present study was to analyze the response of a suite of cellular and biochemical biomarkers in the snail *Cantareus apertus* exposed to mercury through contaminated food. Novel cellular biomarker on heamolymph cells were analyzed, including changes in the spread cell/round cell ratio and haemocyte morphometric alteration. In parallel standardized biomarkers were analyzed such as 1) tissutal metallothioneins (MT) concentration, specific biomarker of exposure to heavy metals, used to establish the activation of detoxification responses against mercury in the studied animals and thus as analytical confirmation of exposure, 2) acetylcholinesterase (AChE) inhibition, which recently has been demonstrated to be sensitive to some metallic ions (for a review see Lionetto et al., 2010), and 3) lysosomal membrane stability, which is routinely used as an early indicator of the adverse effects of contaminants across a wide range of animal species.

C. apertus is a central Mediterranean species that is recorded from France (maritime influenced areas), Italy, and Greece. Outside Europe it is also known from Algeria, Tunisia, and western Libya, and the Mediterranean coast of Turkey, inhabiting all types of maritime influenced Mediterranean habitats. Previous studies demonstrated the suitability of this organism for environmental assessment in in-situ heavy metal pollution (Fritsch et al., 2011) and ex-situ pesticide exposure studies.

C. apertus is also a commercial value species (Avagnina, 2011; Novelli et al., 2002). As reported by Avagnina (2011) only in the year 2011, the quantitative of *C. apertus* eaten was equal to 3.760 tons. Therefore, the assessment of metal induced alterations in this organism could also be of concern for potential effects on human health due to the consumption of this species.

2. Materials and methods

2.1. Material

Adult specimens of *C. apertus* (mean wt 3.475 ± 0.2 g), obtained from a local dealer, were reared in a plastic box (55 × 39 × 25 cm) under controlled conditions of temperature (20 ± 2 °C), photoperiod (18/6 light/dark regime) and humidity (85%) according to De Vaufleury and Gimbert (2009). The floor of the cage was covered with blotting paper, dampened with tap water. Leaves of *Lactuca sativa* were administered ad libitum as food. 120 adult snails were randomly chosen for the exposure experiment and starved for 2 days before starting the experiment.

All chemicals were reagent grade. Diff-Quick® Kit was purchased from Dade Behring, while the other chemicals were purchased from Sigma-Aldrich (St. Luis, MO, U.S.A.).

2.2. Experimental design

A 14 day exposure to $HgCl_2$ through contaminated *L. sativa* was carried out. Three times a week the animals were exposed to *L. sativa*, which had been soaked for 1 h in $HgCl_2$-solutions, according to the method described by Dallinger et al. (2005).Two concentrations were utilized, 0.5 and 1 μM, respectively. Foliar consumption of contaminated leaves is one of the main natural pollutant exposure pathways in terrestrial snails. Mercury is known to be easily absorbed by the leaves from air or water (Niu et al., 2013). In gaseous form, Hg metals can be taken up from the air through the stomata (Gaggi et al., 1991), in ionic form it can be taken up through the cuticle (Greger, 1999).

The concentrations used were below those considered hazardous to humans (TCLP = 0.2 ppm) and were below the LC50 values for acute exposure of aquatic mollusks (Harry and Aldrich, 1963; Meena and Balakrishnan, 1993).

Biomarker responses were monitored in control and treated animals through the time. A three factor experimental design was chosen: factor (A) "mercury exposure" which included three levels ("not exposed" or control animals and "exposed" to 0.5 μM and 1 μM $HgCl_2$), factor (B) "time of exposure" which included four levels (0, 3, 7 and 14 days), and factor (C) "box replication". Five boxes for each condition were utilized and three animals were added in each box (plastic box dimensions: 14 × 10 × 7 cm). All the groups were held in controlled conditions of temperature, photoperiod, and humidity (see above). At any time three animals per box were sampled. Each specimen underwent to haemolymph sampling, performed by a sterilized hypodermic syringe (needle size 26G½: 0.45 mm × 13 mm) through a small hole created on the shell at the hemocoele level according to Regoli et al. (2006). Haemolymph was immediately utilized for cytological staining of haemocytes and for Neutral Red Retention Assay. Then, each snail underwent cold anesthesia (4 °C for 20 min) and was sacrificed. The hepatopancreas were dissected and stored at −80 °C until utilized for metallothionein measurements.

2.3. Haemocyte morphometric analysis

Haemocyte morphometric alterations were determined by image analysis on Diff-Quick® (Dade Behring, Newark, U.S.A.) stained cells, according to the method described by Calisi et al. (2008) and slightly modified. The rapid alcohol-fixed Diff-Quick stain is widely utilized in clinical and veterinary applications for immediate interpretation of histological samples. In recent years it was successfully applied to mussel haemocyte (Calisi et al., 2008) and, also, earthworm coelomocyte staining (Calisi et al., 2009, 2011).

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