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Integrated biomarker analysis in the earthworm *Lumbricus terrestris*: Application to the monitoring of soil heavy metal pollution

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HIGHLIGHTS

- A multi-biomarker response was validated in field in *Lumbricus terrestris*.
- Heavy metal exposure alters earthworm hemoglobin concentration in field.
- Granulocyte size changes were demonstrated as valuable exposure biomarker in field.
- Results provide a sensitive tool for metal pollution monitoring in the soil.

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ABSTRACT

As recently recognized exposure and effect assessment of soil contaminants on soil biota is necessary for decision-making related to ecosystem services and habitat protection, establishment of remediation procedures, or pollution monitoring programs. Therefore, biological approaches to soil monitoring, such as the measurement of biomarkers in soil bioindicator organisms, have recently received increasing attention. The aim of the present work was to assess the performance of a suite of cellular and biochemical biomarkers in native earthworms (*Lumbricus terrestris*) sampled in heavy metal contaminated sites in view of the validation of this biomarker approach in soil monitoring and assessment. Besides well known and standardized biomarkers such as lysosomal membrane stability, metallothionein tissue concentration and acetylcholinesterase activity, novel potential biomarkers such as changes in blood hemoglobin concentration and granulocyte morphometric alterations were analyzed. Both univariate and multivariate (PCA) statistical analysis applied to the data set revealed that the integrated multi-marker approach in native *L. terrestris* under field conditions produces a sensitive and cost-effective assessment of heavy metal soil pollution, which could be incorporated as a descriptor of environmental status in future soil biomonitoring programmes.

1. Introduction

Soil pollution has enormously increased during the last decades because of industrial activities, the intensive use of biocides and fertilizers in agriculture, urban waste and atmospheric deposition. Its occurrence is related to the degree of industrialization and intensity of chemical usage. The risks associated to soil pollution are very severe and represent a threat for living organisms in both belowground and aboveground systems. These risks mainly consist of soil fertility decrease, soil structure alteration, disturbance of the balance between flora and fauna residing in the soil, contamination of the crops, and contamination of groundwater (Lionetto et al., 2012). The most diffuse chemicals occurring in soil are heavy metals, pesticides, petroleum hydrocarbons, polychlorobiphenyl (PCB), dibenzo-p-dioxins/dibenzofurans (PCDD/Fs). In particular heavy metals from anthropogenic sources are widely spread in the environment and most of them finally end up in surface soil layers (Lionetto et al., 2012).

Heavy metals can enter the soil from different sources, such as pesticides, fertilizers, organic and inorganic amendments, mining, industrial emissions and effluents, smelters, wastes and sludge residues (Capri and Trevisan, 2002; Wuana and Okieimen, 2011). In contrast to harmful organic compounds, heavy metals do not decompose and disappear from the soil even if their release to the environment can be restricted (Brusseau, 1997). Therefore, the effects of heavy metal contamination on soil organisms and decomposition processes persist for many years.

As recognized in recent years by international organizations and environmental agencies, soil risk assessment cannot be based solely on chemical analysis of contaminants (Sanchez-Hernandez, 2006) because this approach does not provide an indication of

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deleterious effects of contaminants on the biota. It neglects several essential aspects such as toxicity of chemicals not included in the selection of contaminants to be analyzed, interactive effects (synergism and antagonisms) of pollutants on biota and bioavailability. Considering that bioavailability of pollutant in soil can be influenced by soil pH, cation exchange capacity, and organic matter content, it is difficult to model pollutant bioavailability based on total concentration and soil characteristics alone. The best integrators of these complex effects are the exposed organisms themselves.

Exposure and effect assessment of soil contaminants is therefore necessary for decision-making related to ecosystem services and habitat protection, establishment of remediation procedures, or for impacted sites monitoring programs. For these reasons, new biological approaches to soil monitoring, such as the measurement of biochemical and cellular responses to pollutants (i.e. biomarkers) on organisms living in the soil (i.e., bioindicators), have become of major importance for the assessment of the quality of this environmental compartment (Lionetto et al., 2012).

In this decade, there has been a growing concern in the use of biomarkers in terrestrial invertebrates to assess the potential adverse effects of chemicals on soil ecosystems (Hyne and Maher, 2003; Weeks et al., 2004). The use of molecular and cellular indicators of exposure to, and effects of, contaminants (referred as biomarkers) in field exposed organisms becomes a suitable line of evidence for assessing biological effects from pollution. A biomarker is a biological response (from molecular to behavioral changes) measured in tissue or body fluids samples, or at the level of whole organism, that provides evidence of exposure to and/or toxic effects of one or more chemical pollutants (Depledge et al., 1993; Lagadic et al., 2000; Walker, 2001). Biomarkers can be either specific (monitoring for the exposure/effects of specific chemical classes) or general (monitoring for a generic stress syndrome, due to the integrated effect of several contaminant classes).

Earthworms are an essential component of the soil fauna, and represent a significant proportion of the soil biomass (Bouche, 1992; Sizmur and Hodson, 2009). They contribute to pedogenesis and soil profile influencing physical, chemical and microbiological properties of the soil (Barlett et al., 2010). Earthworms give a fundamental contribution to decomposition of organic matter and subsequent cycling of nutrients. Therefore, these animals are regarded as useful indicators of soil health and quality (Edwards, 2004). This has contributed to their use as bioindicator organism of soil pollution (Spurgeon et al., 2003). In recent years the study of biomarkers in earthworms has received increased attention (Reinecke and Reinecke, 2004; Van Gestel and Weeks, 2004; Sanchez-Hernandez, 2006). In particular, biomarker analysis on native earthworm populations offers the advantage of an ecologically more relevant approach to environmental monitoring and assessment (Sanchez-Hernandez, 2006). In addition the usefulness of native organisms arises mainly when studying pollutant long-term effects that may be emphasized in organisms from natural populations. In fact it is difficult to extrapolate effects from spiked soils to field soils, when these are already polluted for a long time (Lionetto et al., 2012). The bioavailability of pollutants in comparable soil types polluted in the field or spiked in the lab is different (Smolders et al., 2003). Soil characteristics, e.g. pH, organic matter and clay content (Peijnenburg, 2002) also play an important role in determining the bioavailability of pollutants. Using native earthworm populations for biomarker analysis integrates the bioavailability of pollutants, exposure pathways and temporal aspect of exposure (Spurgeon et al., 2002; Sanchez-Hernandez, 2006). In spite of the recognized importance of biomarker study in native populations, so far only few studies (Hankard et al., 2004; Weeks et al., 2004) have explored the potentiality of the biomarker approach in native earthworms, if compared with studies on aquatic environments.

The aim of the present work was to assess the performance of a suite of cellular and biochemical biomarkers in native earthworms (*Lumbricus terrestris*) sampled in heavy metal contaminated sites in view of the validation of this biomarker approach in soil monitoring and assessment. Besides well known and standardized biomarkers such as lysosomal membrane stability, metallothionein tissue concentration and acetylcholinesterase activity, novel potential biomarkers such as changes in blood hemoglobin concentration and granulocyte morphometric alteration were analyzed. These responses have been recently identified in earthworms as sensitive responses to heavy metal exposure in laboratory exposure conditions (Calisi et al., 2009, 2011a,b). *L. terrestis* was chosen as autochthonous species inhabiting the study sites. It is an anecic earthworm that forms deep burrows and comes to the surface to feed. This earthworm species has a large size which permits handling and collection of enough amounts of fluid and tissues for biomarker analysis (Diogene et al., 1997; Affar et al., 1998).

2. Materials and methods

2.1. Sampling

The earthworm sampling was carried out in four sites in Southern Apulia (Italy): two impacted Sites of National Interest (D.L. 426, 1998), "Ilva" (Taranto) and "Cerano" (Brindisi) (Fig. 1), and two control sites Aradeo (LE) and Erchie (BR). The Ilva area is impacted by the activities of a large heavy industry site. Particular attention is turned to the presence of a steelworks which represents one of the biggest steel manufacturer plants in Europe. The Cerano area is impacted by the presence of an active carbon-electric central. Aradeo and Erchie are not interested by industrial or intensive agriculture activities. They were chosen as control sites.

Ten adult and clitellated earthworms (*L. terrestris*) were randomly collected in uncultivated soil in the four sites. In ILVA and Cerano sites the sampling was performed in soils adjacent to the industrial area. Individuals of the same size were selected to ensure uniform sampling. The body weight of depurated – void gut – earthworms was respectively: 2.10671 ± 0.3165 (Cerano), 2.1053 ± 0.464996 g (Ilva), 2.26529 ± 0.299443 Erchie, 2.24384 ± 0.344971(Aradeo). Coelomic fluid and blood samples were obtained from each collected specimen. Then, the animals were frozen at −80 °C until utilized for metallothionein and acetylcholinesterase measurement. Coelomic fluid was obtained by puncturing postclitellum's segments of the coelomic cavity by a sterilized hypodermic syringe and immediately utilized for cytological staining of coelomocytes and for neutral red retention assay. Blood was obtained from the ventral vessel by puncturing pre-clitellum's segments with a sterilized hypodermic syringe with a 30 G thin needle. The blood sample was immediately used for hemoglobin determination.

2.2. Granulocyte morphometric analysis

Granulocyte morphometric alterations were determined by image analysis on Diff-Quick® (Dade Behring, Newark, USA) stained cells as reported by Calisi et al. (2009). The rapid alcohol-fixed Diff-Quick stain is widely utilized in clinical and veterinary applications for immediate interpretation of histological samples. Recently it was successfully applied to mussel hemocytes (Calisi et al., 2008) and earthworm coelomocyte staining (Calisi et al., 2009, 2011a).

A volume (40 μL) of hemolymph (diluted 1:1 in a saline solution containing 10 mM N-[hydroxyethyl]piperazine-N′-[2-ethanesulfo-