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Effects of dioxin exposure in *Eisenia andrei*: integration of biomarker data by an Expert System to rank the development of pollutant-induced stress syndrome in earthworms

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ABSTRACT

A battery of biomarkers has recently been developed in the earthworm *Eisenia andrei*. In this study, different biomarkers (i.e. Ca^{2+}-ATPase activity, lysosomal membrane stability-LMS, lysosomal lipofuscin and neutral lipid content) were utilized to evaluate the alterations in the physiological status of animals, induced by exposure for 3 d to different sublethal concentrations of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) (1.5×10^{-3}, 1.5×10^{-2}, 1.5×10^{-1} ng mL^{-1}) utilizing the paper contact toxicity test. Lysosome/cytoplasm volume ratio and DNA damage were also evaluated as a biomarker at the tissue level and as a biomarker of genotoxicity, respectively. Moreover, the NR retention time assay conditions were optimized for the determination of in vivo LMS in earthworm coelomocytes. The results demonstrate that LMS and Ca^{2+}-ATPase activity were early warning biomarkers able to detect the effects of minimal amounts of TCDD and that biomarkers evaluated at the tissue level are important for following the evolution of the stress syndrome in earthworms. To evaluate the health status of the animals, an Earthworm Expert System (EES) for biomarker data integration and interpretation was developed. The EES proved to be a suitable tool able to rank, objectively, the different levels of the stress syndrome in *E. andrei* induced by the different concentrations of TCDD.

1. Introduction

Over recent decades, earthworms (Anellida, Oligochaeta) have been extensively used as model organisms for assessing the toxic effects of contaminants in soils (Spurgeon et al., 2003; Lee et al., 2008). These invertebrates do indeed represent one of the meaningful targets of potential soil toxicity as they are in direct and constant contact with the particles through both their external and internal surfaces (Jager et al., 2003; Lanno et al., 2004). As a representative group of macrofauna in most soils worldwide, in terms of both abundance and cosmopolite spread, and due to their ecological role in the soil habitat, these organisms represent key species within terrestrial ecosystems (Lee, 1985; Römbke et al., 2005).

The species *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* (Bouché, 1972) (Oligochaeta, Lumbricidae) are the most commonly used in ecotoxicology (OECD, 1984, 2004; ISO, 1993, 1998, 2008). Their features, such as world-wide distribution, susceptibility to chemicals, wide temperature and moisture tolerance range, short life cycles and the fact that they can be easily cultivated, make these earthworm species suitable for use in toxicological studies (Greig-Smith et al., 1992; Domínguez, 2004).

In addition to mortality and reproduction, as typical ecotoxicological high-level endpoints, earthworm biomarkers are the object of increasing interest for the assessment of the potential toxicity of chemicals (Scott-Fordsmand and Weeks, 2000; Sanchez-Hernandez, 2006). Biomarkers are able to reveal alterations, caused by pollutants, in the physiological status of organisms, thus providing sensitive early-warning responses (Depledge and Fossi, 1994; Moore et al., 2004).

A battery of biomarkers has recently been developed in *E. andrei*. In laboratory experimental conditions, earthworm exposure to sublethal concentrations of both heavy metals and polycyclic aromatic hydrocarbons induced significant changes in all the studied biomarkers of stress, i.e. Ca^{2+}-ATPase activity, lysosomal membrane stability (LMS), lysosomal lipofuscin and neutral lipid content (Gastaldi et al., 2007). Moreover, in earthworms, metallothionein content and acetylcholinesterase activity were shown to represent sensitive biomarkers of exposure to heavy metals and pesticides, respectively (Caselli et al., 2006; Gastaldi et al., 2007).

The determination of multiple biomarkers across different levels of functional complexity, from the most sensitive molecular changes to whole-organism responses, is an essential approach to providing evidence of the development of the pollutant-induced stress syndrome in the organisms (Moore et al., 1987; Depledge et al., 1995; Cajaraville et al., 2000; Kammenga et al., 2000;

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Spurgeon et al., 2005; Viarengo et al., 2007). Nevertheless, from the simple analysis of the simultaneous changes of different physiological parameters, it is generally difficult to obtain a correct evaluation of the overall changes in the health status of the organisms induced by pollutant exposure. The development of bioinformatic tools able to integrate information derived from different biological data into synthetic stress indices should allow an objective interpretation of ecotoxicological data (Narbonne et al., 1999; Viarengo et al., 2000; Beliaeff and Burgeot, 2002; Moore et al., 2004).

An algorithm for the integration and interpretation of biomarker data, the Mussel Expert System (MES), has recently been developed for the marine bivalve *Mytilus* spp. (Dagnino et al., 2007). The MES allows for a correct selection of parameters at different levels of biological organization (molecular/cellular/tissue/organism), taking into account trends in pollutant-induced biomarker changes (increasing, decreasing, bell-shaped). On the basis of this information and of the physiological meaning of the different biomarkers employed, the MES also considers the possible mutual interferences that may occur among various biological responses under stress conditions. The MES proved to be a reliable and easy-to-use tool able to rank the levels of pollutant-induced stress syndrome in mussels (Dondero et al., 2006; Dagnino et al., 2007; Franzellitti et al., 2010; Raftopoulou and Dimitriadis, 2010).

In this work, physiological parameters at different levels of functional complexity were evaluated in *E. andrei* in order to assess the evolution of the pollutant-induced stress syndrome in the earthworms. The results were then utilized for the development of expert-system procedures for biomarker data management to evaluate the health status of the animals. To this end, earthworms were exposed for 3 d to different sublethal concentrations of a model Persistent Organic Pollutant (POP), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic member of the dioxins (Safe, 1986; Whitlock, 1990), utilizing the paper contact toxicity test (OECD, 1984). In this study, we utilized Ca^{2+}-ATPase activity in the intestinal epithelium, and the lysosomal lipofuscin and neutral lipid content in the cells of the chloragogenous tissue as biomarkers at molecular/cellular level; in this latter tissue, the lysosome/cytoplasm volume ratio was also investigated as a biomarker suitable for demonstrating pollutant-induced alterations at tissue level. Moreover, a modified NR retention time assay was applied to optimize the *in vivo* determination of LMS in earthworm coelomocytes. DNA damage of the coelomocytes was also evaluated by the Comet assay as a biomarker of potential genotoxicity. The obtained results were utilized to develop an Earthworm Expert System (EES) of integration and interpretation of biomarker data suitable for ranking the different phases of the evolution of the stress syndrome in *E. andrei* induced by pollutant exposure.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated.

2.2. Animals

Earthworms were cultured essentially as described in the OECD guidelines (OECD, 1984, 2004), i.e. in a breeding medium made up of a mixture of horse manure and peat. Breeding was carried out in a climatic chamber at 20 ± 1 °C. The animals were fed using air-dried finely ground horse manure and oatmeal. Organisms were selected from a synchronized culture with an homogeneous age structure. Adult worms with clitellum of similar size and weight (of 400–500 mg) were utilized in the experiment.

2.3. Paper contact toxicity test

The filter paper test was performed as described in the OECD guideline for the testing of chemicals (OECD, 1984). Worms were kept on clean moist filter paper for 3 h before being placed in test dishes to allow them to void their gut contents. Animals were then washed with deionised water and dried before use. Dioxin was dissolved in acetone to give the range of concentrations used in the experiment, i.e. 1.5×10^{-3}, 1.5×10^{-2}, 1.5×10^{-1} ng mL^{-1}. Then, 1 mL of each contaminant solution was spread onto a filter paper (Whatman grade 1), evaporated to dryness and placed on the bottom of a Petri dish. Control filter papers were treated with 1 mL of acetone. After drying, 1 mL of deionised water was added to each dish to moisten the filter paper. The dishes were put in a climatic chamber with a temperature of 20 ± 1 °C. The test was performed in the dark and for a period of 3 d. At least five replicates per treatment for each assay, consisting of one worm per dish, were used.

Animals were weighed before the start and at the end of the experiments.

2.4. Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity in the intestinal epithelium was assessed on resin-embedded tissue sections (2 μm) by the histochemical method described by Gastaldi et al. (2007). Slides were observed using an inverted microscope (Zeiss Axiovert 100M) at 400× magnification, connected to a digital camera (Zeiss AxioCam). The pictures obtained were analysed using an image analysis system (Scion Image freeware) that allowed for the quantification of changes in enzymatic activity, that were expressed as a percentage of optical density with respect to controls.

2.5. Lipofuscin and neutral lipid lysosomal content

Lipofuscin and neutral lipid lysosomal content in the cells of the chloragogenous tissue were estimated on cryostat sections (10 μm) as previously described (Gastaldi et al., 2007). The lipofuscin content was assessed using the Schmorl reaction (Pearse, 1972; Moore, 1988). Neutral lipid content was evaluated by Oil Red-O (ORO) staining (Moore, 1985). Lipofuscin and neutral lipid accumulations were quantified by image analysis as described above and expressed as a percentage variation with respect to controls.

2.6. Lysosomal membrane stability

Lysosomal membrane stability of coelomocytes was evaluated utilizing a modified version of the method previously developed by Gastaldi et al. (2007). Coelomocytes obtained by the extrusion method (Eyambe et al., 1991; Fugère et al., 1996) were placed on polylysinated slides where they were allowed to adhere for 15 min in a humidity chamber at 20 ± 1 °C. The cells were then incubated in a working solution of neutral red (NR), obtained by diluting 10 μL of a stock solution of NR (20 mg of NR in 1 mL of DMSO) with 990 μL of Hanks' Balanced Salt Solution (HBSS) (Sigma product H8264). After 5 min, excess dye was eliminated and the cells were washed and kept moist with HBSS. The retention time of NR dye within the lysosomes (NRRT) was monitored after 1 h. Slides were viewed under 630× magnification by an inverted photo-microscope (Zeiss Axiovert 100M) equipped for fluorescence microscopy using a rhodamine emission filter. Images were analysed using an image analysis system as described above that allowed for the quantification of the lysosomal NR leakage, that was expressed as a percentage change in fluorescence intensity with respect to controls.