Ecotoxicology and Environmental Safety 72 (2009) 1369–1377

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Pollutant-induced alterations of granulocyte morphology in the earthworm *Eisenia foetida*

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ARTICLE INFO

Article history:
Received 24 September 2008
Received in revised form
11 March 2009
Accepted 15 March 2009
Available online 2 May 2009

Keywords:
Earthworms
Biomarkers
Coelomocytes
Granulocytes
Morphometric alterations

ABSTRACT

Earthworms are considered convenient indicators of land use and soil fertility. Recently the use of biomarkers in earthworms has been increasingly investigated. The aim of this work was to study possible pollutant-induced morphometric alterations in *Eisenia foetida* granulocytes in view of future applications as a sensitive, simple, and quick biomarker for soil monitoring and assessment applications. Results showed consistent enlargement of earthworm granulocytes induced by exposure to either copper sulfate or methiocarb. The increase of cellular size was time-dependent and was about 100% after 14 days of exposure for both treatments. In order to verify the applicability of morphometric granulocyte alteration, a battery of standardized biomarkers such as lysosomal membrane stability, metallothionein induction, or acetylcholinesterase (AChE) inhibition were also determined. We recommend the use of morphometric alterations of granulocytes as a suitable biomarker of pollutant effect to be included in a multibiomarker strategy including responses at different levels of biological organization.

1. Introduction

As recognized in recent years by international organizations and environmental agencies, soil risk assessment cannot be based solely on chemical analysis of contaminants in the soil compartment (Kammenga et al., 2000) because this approach does not provide an indication of deleterious effects of contaminants on the biota. The need to detect and assess the effects of contamination at low concentrations and in complex mixtures has recently led to the development of molecular and cellular indicators of exposure to and effects of contaminants (commonly referred as biomarkers) in field-exposed organisms. A biomarker is a biochemical, cellular, physiological, or behavioral variation measured in tissue or body fluids samples, or at the level of whole organisms, that provides evidence of exposure to and/or toxic effects of one or more chemical pollutants (Depledge et al., 1993). Biomarker responses can be either specific (monitoring for the presence/effects of specific chemical classes) or general (monitoring for a generic stress syndrome, due to the integrated effect of several contaminant classes).

Earthworms are very important organisms for soil formation and organic matter breakdown in most terrestrial environments, and traditionally they have been considered to be convenient indicators of land use and soil fertility (Paoletti, 1999). Because of their strong interaction with soil, earthworms are profoundly affected by soil pollution coming from intensive use of biocides in agriculture, industrial activities, and atmospheric deposition. These features, among others, have led to the use of earthworms as bioindicators of soil pollution (Cortet et al., 1999; Lanno et al., 2004). In recent years the use of biomarkers in earthworms has received increased attention. There is a growing interest in increasing the knowledge of biological responses of earthworms to pollutants in order to standardize a suite of biomarkers covering molecular to whole-organism endpoints in response to soil chemical pollution (Beliaeff and Burgeott, 2002; Handy et al., 2003).

The coelomic fluid of earthworms has a function analogous to that of blood in the sense that this fluid medium is responsible for pollutant disposition and tissue distribution. Its cells, coelomocytes, are responsible for the immune defense of the animal (Cooper et al., 1995, 2002; Engelmann et al., 2004; Reinhart and Dollahan, 2003). Therefore, any impairment of coelomocyte functioning can compromise the health of the entire organism. To date the most investigated coelomocyte alteration is represented by lysosomal membrane stability, used as an indicator of chemical exposure and associated biological effects (Maboeta et al., 2002; Svendsen et al., 1996, 2004).

The aim of the present work was to investigate possible pollutant-induced morphometric alterations in earthworm coelomocytes in view of future application as sensitive, simple and quick biomarker for soil monitoring and assessment applications. The study was carried out on the earthworm *Eisenia foetida*, which

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doi:10.1016/j.ecoenv.2009.03.010

is widely used for toxicity bioassays (Bouché, 1992; ISO, 1993, 1996, 1999) and biomarker application in soil biomonitoring (Scott-Fordsman and Weeks, 2000; Kammenga et al., 2000). The animals were exposed in controlled laboratory conditions either to the heavy-metal-based fungicide copper sulfate or to the carbamate methiocarb (4-methylthio-3,5-xylyl methylcarbamate). Carbamate pesticides have become one of the most widely used pesticides today, and they represent an important group of pesticides in the authorized list of agrochemicals in the EEC (Council Directive) (1991). In general they have a low persistence in the environment; however, many of them show high acute toxicity for the soil biota. Copper sulfate has many agricultural uses as a fungicide and molluscicide. In addition, copper and other heavy metals can enter the soil from other sources, such as fertilizers, organic and inorganic amendants, wastes, and sludge residues (Capri and Trevisan, 2002). Carbamate and copper sulfate were selected as model toxicants of different classes of chemical pollutants relevant to soil risk assessment.

To our knowledge this is the first time that morphometric coelomocyte alteration in earthworms has been proposed as a biomarker of pollutant exposure in the soil. In order to verify the applicability of morphometric coelomocyte alteration in a biomarker battery on the bioindicator organism *E. foetida*, standardized biomarkers such as lysosomal membrane stability (general biomarker), metallothioneins (MTs) (biomarker of exposure to heavy metals), and acetylcholinesterase (AChE) (biomarker of exposure/effect to organophosphate and carbamate pesticides) were also determined.

2. Materials and methods

All chemicals were reagent grade. Diff-Quick was purchased from Dade Behring; all the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Experimental design

Mature earthworms (*E. foetida*), collected at the Botanic Garden of the University of Salento, were utilized for the laboratory exposures. A homogeneous stock of earthworms (*E. foetida*) ($n = 120$) of the same size (body weight of depurated—void gut—earthworms was 0.38 ± 0.04 g) was acclimated for 48 h in boxes (40 cm × 18 cm × 16 cm) containing 3 kg of aerated soil under controlled conditions: 18 ± 1 °C, 40% humidity, and 16/8 h light/dark regime. The soil was a mixture of peat (60%), sand (23%), and silt (17%). After the acclimation period, the stock was divided into two groups: the first ($n = 60$) was utilized for the exposure experiment with copper sulfate, the second ($n = 60$) for the exposure experiment with methiocarb.

For each exposure experiment, a three-factor experimental design was chosen: factor (A), "toxicant exposure," included two levels ("not exposed" or control animals and "exposed"), factor (B), "time of exposure," included three levels (0, 14, and 30 days of exposure for copper sulfate, 0, 7, and 14 days of exposure for methiocarb), and factor (C), "box replication," included two levels (two boxes for each condition were utilized). Factors A and B were fixed and orthogonal to each other, while Factor C was nested in the combination A × B. Eight animals were added to each box replicate. The toxicant concentrations utilized were 140 mg/kg copper sulfate and 100 mg/kg methiocarb (1%), respectively. They represent the maximal concentrations recommended in vineyards (CEC, 1986; EEC, 2001; Paoletti et al., 1998). The toxicants were dissolved in water and added to the terrain at the start of the exposure experiment. The duration of copper sulfate exposure was 30 days, while the duration of methiocarb exposure was 14 days. The choice of the different times of exposure arises from the different half-lives of these two compounds in the soil. DT50 of methiocarb is estimated to be 8 days in soils, although a longer half-life has been observed in indoor studies (Keum et al., 2000). On the other hand, copper does not degrade, but simply can change its speciation in soil.

All the groups were held in controlled conditions (see above). At any time eight animals per box were sampled. Each specimen was weighed and subjected to coelomic fluid sampling. Then the animals were frozen at −80 °C until utilized for metallothionein and acetylcholinesterase measurement.

2.2. Cytological biomarkers

Coelomic fluid was obtained by puncturing post-clitellum segments of the coelomic cavity with a sterilized hypodermic syringe with a gentle and deliberate drawing action and immediately utilized for cytological staining of coelomocytes and for neutral red retention assay (NRRA).

2.2.1. Morphometric analysis

Coelomocyte morphometric alterations were determined by image analysis on Diff-Quick (Dade Behring, Newark, USA) stained cells. The rapid alcohol-fixed Diff-Quick stain takes less than 5 min and results in excellent cytological detail, comparable to that with Papanicolaou stain (Hirschowitz et al., 1994). For this reason it is widely utilized in clinical and veterinary applications for immediate interpretation of histological samples. Recently it was successfully applied to mussel hemocyte staining (Calisi et al., 2008). In this work, for the first time, the Diff-Quick staining protocol was shown to be suitable for cytological staining of earthworm coelomocytes also.

A volume (40 μl) of hemolymph (diluted 1:1 in a saline solution containing 10 mM *N*-[hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 125 mM NaCl, 0.4 mM $MgSO_4$, 2.7 mM KCl, and 1.8 mM $CaCl_2$, pH 7.4 with NaOH 1 M) was dispensed on a poly-L-lysine-coated slide, incubated in a humid chamber (16 °C) for 30 min, and stained with the Diff-Quick kit. Samples were fixed and stained on slides by repeated 1-s dips in the three reagents of the Diff-Quick kit in sequence: fast green (fixative) (five dips), eosin G in phosphate buffer pH 6.5 (18 dips), and thiazin dye in phosphate buffer pH 6.5 (two dips). subsequently they were washed in distilled water and air-dried. Diff-Quick stained coelomocytes were observed by an optical microscope (Eclipse E600, Nikon, Tokyo, Japan) and the images obtained from a video camera (TK-C1381, JVC, Yokohama, Japan) were digitalized using the LUCIA image analysis software (Nikon, Tokyo, Japan). The cell area and the lysosomal compartment area of 2-D digitized coelomocyte images were automatically calculated by the LUCIA software. Approximately 80 cells per sample were analyzed.

2.2.2. NRRA

The NRRA method used in the present study has been described previously by Weeks and Svendsen (1996). Briefly, 40 μl of coelomic fluid (diluted 1:1 as above) were dispensed on a poly-L-lysine-coated slide and incubated in a humid chamber (16 °C) for 30 min. Forty microliters of neutral red solution (995 μl of saline solution and 5 μl of neutral red stock solution obtained by dissolving 20 mg of neutral red powder in 1 ml dimethyl sulfoxide) were added and the slides were left in a humid chamber (16 °C) for 15 min. Then a cover slide was applied and the slide was observed under the microscope. The slides were observed every 15 min for the first hour and every 30 min for