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Species-specific differences in biomarker responses in two ecologically different earthworms exposed to the insecticide dimethoate

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ABSTRACT

Earthworms ingest large amounts of soil and therefore are continuously exposed to contaminants through their alimentary surfaces. Additionally, several studies have shown that earthworm skin is a significant route of contaminant uptake as well. In order to determine effects of dimethoate, a broad-spectrum organophosphorous insecticide, two ecologically different earthworm species were used — *Eisenia andrei* and *Octolasion lacteum*. Although several studies used soil organisms to investigate the effects of dimethoate, none of these studies included investigations of dimethoate effects on biochemical biomarkers in earthworms. Earthworms were exposed to 0.001, 0.005, 0.01, 0.5 and 1 μg/cm^2 of dimethoate for 24 h, and the activities of acetylcholinesterase, carboxylesterase, catalase and efflux pump were measured. In both earthworm species dimethoate caused significant inhibition of acetylcholinesterase and carboxylesterase activities, however in *E. andrei* an hormetic effect was evident. Efflux pump activity was inhibited only in *E. andrei*, and catalase activity was significantly inhibited in both earthworm species. Additionally, responses of earthworm acetylcholinesterase, carboxylesterase and catalase activity to dimethoate were examined through *in vitro* experiments. Comparison of responses between *E. andrei* and *O. lacteum* has shown significant differences, and *E. andrei* has proved to be less susceptible to dimethoate exposure.

1. Introduction

Earthworms are considered as soil engineers because of their ability to modify soils and plant communities (Lavelle et al., 1997; Hale et al., 2005). As earthworms ingest large amounts of soil or specific fractions of soil (i.e., organic matter), they are continuously exposed to contaminants through their alimentary surfaces (Morgan and Morgan, 1988) and therefore are profoundly affected by agricultural practices, such as soil tillage, crop residues, and the use of fertilizers and pesticides (Edwards, 1983; Lofs-Holmin, 1983; Daugbjerg et al., 1988). Also, several studies have shown that earthworm skin is a significant route of contaminant uptake as well (Saxe et al., 2001; Jager et al., 2003; Vijver et al., 2003). Earthworms can be divided into three main ecological groups; (1) epigeic species (pigmented, living superficially in the litter layer, form no permanent burrows and feed on decaying organic matter and litter materials), (2) endogeic species (living in horizontal burrows at approximately 10–15 cm depth and feed on the organic matter in the soil) and (3) anecic species (relatively large worms, living in vertical burrows from which they collect dead organic matter on the surface at night) (Bouché, 1977). Since earthworms in different functional groups have different activities and feeding behaviors, in the present study two ecologically different earthworm species were used. The epigeic species *Eisenia andrei* was chosen since it is particularly appropriate as a test organism for the evaluation of environmental contamination and chemical testing under laboratory conditions (Labrot et al., 1996; Salogovic et al., 1996; Ville et al., 1997). Additionally, *E. andrei* is a test species recommended by OECD (OECD, 1984). Sensitivity tests of multiple earthworm species have showed that *E. andrei* and *E. foetida* are less sensitive species (Ma and Bodt, 1993; Kula, 1995; Fitzgerald et al., 1996), so in toxicity testing this should be taken into account. The second earthworm species used in this study was endogeic species *Octolasion lacteum*. This species belongs to a different ecological group, it is widely distributed over the world and none of the studies that used *O. lacteum* investigated effects of pesticides.

Dimethoate (O, O — dimethyl S — methylcarbamoylmethyl phosphorodithioate) is a broad-spectrum organophosphorous insecticide that was introduced in 1956 to combat a wide range of insect pests in agriculture, as well as houseflies (WHO, 1989). Dimethoate is one of the most used organophosphate insecticides in the European Union member states, with a consumption of 581 t of active substance in 2003 (Eurostat, 2007). Organophosphorus compounds (OPs), as well as their active metabolites, are inhibitors of serine esterases, i.e., acetylcholinesterase (AChE), butyrylcholinesterase (BChE), neuropathy target esterase (NTE) and carboxylesterase (CES) (Fukuto, 1990; Wheelock et al., 2005; Masson and Lockridge, 2010; Satoh and Gupta, 2010). The inhibition of these enzyme activities is due to phosphorylation of the

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active-site serine. Measurement of inhibition of AChE activity is routinely used as a biomarker of exposure to organophosphate compounds (Reinecke and Reinecke, 2007). CES are also used as biomarkers of OP exposure; these enzymes play an important role in pesticide detoxification and they irreversibly bind (1:1 ratio) to OP insecticides (Maxwell, 1992). Moreover, phosphorylated AChE and CES activities in earthworms display extremely slow recovery rate (Aamodt et al., 2007; Rault et al., 2008; Collange et al., 2010). Besides the inhibition of serine esterases, some OP insecticides are able to induce oxidative stress (Lukaszewicz-Hussain, 2010), either by overproduction of free radicals (most commonly reactive oxygen species, ROS) or by alteration in antioxidant defense mechanisms, including detoxification and scavenging enzymes (Abdollahi et al., 2004). Catalase (CAT) is one of the essential enzymes in the process of ROS detoxification and plays a key role in the cellular antioxidant mechanism. A change in CAT activity is an indicator of a cellular lesion after exposure to chemicals, and thus it is considered as an early environmental stress biomarker in most cases (Gao et al., 2008). Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment and they are found in both prokaryotes and eukaryotes (Bambeke et al., 2003). Few studies have shown that structurally diverse pesticides with specific physical/chemical determinants have a potency to modulate the transport activity of P-glycoprotein (P-gp) efflux pump in P-gp expressing cell lines at high concentrations (100 mM) (Bain and LeBlanc, 1996; Bain et al., 1997), thus it is possible that they could also modulate the activity of other types of efflux pumps. Recently Hackenberger et al. (2012) provided the evidence for the efflux pump activity in the earthworm *E. andrei* and in order to understand the mechanism(s) of pesticide toxicity it is useful to determine the effects of investigated pesticides on efflux pumps which could play an important role in the defense of the organism. Additionally, since efflux pump may prevent the accumulation of harmful compounds in cells, such as OP, it can greatly impact the final harmful effects (Hackenberger et al., 2012).

Past studies have investigated the toxic effects from dimethoate on the life cycle traits of earthworms. Martikainen (1996) found that biomass reduction of the earthworms occurred at lower concentrations than reduction in survival. Yasmin and D'Souza (2007) investigated dimethoate effects on *E. fetida* and found a significant reduction in the earthworm growth in a dose-dependent manner. In the study of Loureiro et al. (2005), earthworms showed a significant response against dimethoate-contaminated soils, and Dalby et al. (1995) recorded a weight loss of earthworms in response to dimethoate. This OP insecticide showed a considerable acute toxicity especially to the indigenous earthworm species while sublethal effects occurred at low concentrations (Kula and Larink, 1997). Santos et al. (2011a, 2011b) used a small-scale terrestrial ecosystem that could mimic pesticides exposure in the field and the study showed that exposure of earthworms to dimethoate caused a decrease in their weight, although the decrease was not statistically significant. However, none of these studies included investigations of dimethoate effects on biochemical biomarkers in earthworms. The utility of biochemical approaches in environmental pollution monitoring and characterization of effect/exposure to stressor for the use in environmental risk assessment is based on the assumption that low concentrations of a toxicant will cause biochemical responses within individual organisms before these effects are observed at higher levels of biological organization (Sarkar et al., 2006). Even if these biomarkers were not able to provide reliable predictions of effects at higher levels of biological organization, they may reveal the mechanisms underlying these effects (Forbes et al., 2006). The use of biochemical biomarkers could contribute to better understanding of the mechanism of toxic action of environmental pollutants and biomarkers selected in this study could be used in subsequent investigations of toxicokinetic of OP insecticides in earthworms. So the main objectives of this study were to evaluate the biochemical responses in two ecologically different earthworm species and to compare the results between these two species. An experimental study was conducted using filter contact paper test and four biochemical biomarkers were measured — AChE activity, CES activity, CAT activity and efflux pump activity. Additionally, responses of earthworm AChE, CES and CAT activities to dimethoate were examined through *in vitro* experiments.

2. Materials and methods

2.1. Earthworms

The adult *E. andrei* earthworms (Oligochaeta, Lumbricidae) were obtained from the culture maintained in our laboratory with cow dung as a substrate and food. The earthworms were removed from culture, rinsed with tap water, and placed in Petri dishes on damp filter paper for 24 h (in the dark at 20 °C) to void the gut contents. The earthworms used in this assay were all adults with well-developed clitellae — 0.21 ± 0.04 g (mean ± standard deviation of 279 earthworms after voiding the gut content).

Specimens of *O. lacteum* earthworms (Oligochaeta, Lumbricidae) were collected by hand-sorting from uncontaminated grove. In order to establish the earthworm culture, earthworms were kept in a natural agricultural soil that was sampled from the uncontaminated environment, sterilized, griddled and placed in cylindrical pipes 1 m in height and 30 cm in diameter. After 6 months the earthworms were removed from the soil, rinsed with tap water, and placed in Petri dishes on damp filter paper for 24 h (in the dark at 17 °C) to void the gut contents. The earthworms used in this assay were all adults with well-developed clitellae — 0.41 ± 0.09 g (mean ± standard deviation of 279 earthworms after voiding the gut content).

2.2. Chemicals

All reagents used in the study were of analytical grade. 5,5′dithiobis-2 nitrobenzoic acid (DTNB), acetylthiocholine iodide (AcSChI), 4-nitrophenyl acetate, bovine serum albumin (BSA) and rhodamine B (RB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chromgor 40 (Chromos Agro d.d., Zagreb) was the commercial formulation of dimethoate used in the experiments. This formulation contains 400 g/L of active ingredient.

2.3. Filter paper contact test

We compared the toxic responses of *E. andrei* and *O. lacteum* to dimethoate using the *in vivo* paper contact test (OECD, 1984). In the preliminary mortality tests earthworms were exposed to 0.1, 0.5, 1, 2.5, 5, 7.5, 10, 15, 25 and 50 μg/cm^2 of dimethoate and the sublethal concentrations for subsequent experiments were chosen based on results obtained with *E. andrei*. Earthworms were exposed to 0.001, 0.005, 0.01, 0.5 and 1 μg/cm^2 of dimethoate for 24 h at 20 °C. The recommended agricultural application of dimethoate is 1 L of commercial solution (i.e. 400 g of active ingredient) per 1 ha which corresponds to the concentration of 4 μg/cm^2, so the concentrations tested in this study are similar or below the expected concentrations in the soil after the recommended application rate. Dimethoate was suspended in distilled water and loaded onto the filter paper in a flat-bottom glass vials 4.4 cm in length and 5 cm in diameter (2 mL of solution per vial). Controls were also run in parallel with distilled water only. One earthworm was placed in each vial closed with a cap with a ventilation hole and kept in the dark. Experiments were conducted in triplicates with 7-earthworms/replicate. If mortality of occurred during the exposure, the measurements were conducted on survived earthworms. After time of exposure ended, whole earthworms with addition of cold phosphate buffer (0.1 M, pH 7.2) (1:5 w:v for *E. andrei* and 1:4 w:v for *O. lacteum*), were homogenized on ice. The homogenates were then centrifuged for 30 min at 9000 *g* and 4 °C to yield the post-mitochondrial fraction.