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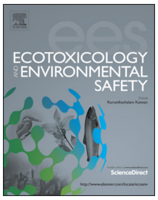

Application of microcosmic system for assessment of insecticide effects on biomarker responses in ecologically different earthworm species

Mirna Velki, Branimir K. Hackenberger*, Željka Lončarić, Davorka K. Hackenberger

Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia

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ABSTRACT

Earthworms from different ecological categories—epigeic *Eisenia andrei* and *Lumbricus rubellus*, endogeic *Octolasion lacteum* and anecic *Lumbricus terrestris*—were exposed in a microcosmic system to three commonly used insecticides. The effects of the insecticides were evaluated by measuring the following molecular biomarkers—the activities of AChE, CES, CAT, GST and the concentration of GSH. The results showed that environmentally relevant doses of organophosphates dimethoate and pirimiphos-methyl significantly affected the measured biomarkers, whereas pyrethroid deltamethrin did not affect the earthworms at the recommended agricultural dose. Considering the ecological category of earthworms, the results were inhomogeneous and species-specific differences in the biomarker responses were recorded. Since the biomarker responses of the investigated earthworm species were different after exposure to organophosphates in a microcosm compared to the exposure via standardized toxicity tests, two types of species sensitivity should be distinguished—physiological and environmental sensitivity. In addition, the hormetic effect of organophosphates on AChE and CES activities was recorded. The detection of hormesis in a microcosm is of great importance for future environmental research and soil biomonitoring, since in a realistic environment pollutants usually occur at low concentrations that could cause a hormetic effect. The results demonstrate the importance of the application of microcosmic systems in the assessment of the effects of environmental pollutants and the necessity of taking into account the possible differences between physiological and environmental species sensitivity.

1. Introduction

Earthworms are a suitable indicator species for the ecotoxicological assessment of soil pollution from pesticides (Schreck et al., 2008) and their use in ecotoxicological and toxicological studies has become more common, especially their utilization as standard test organisms—standardized tests using earthworms are an integral part of the legislation, and part of the European Union directives (Regulation (EC) 1107/2009) for environmental protection. Based on different morphological and behavioral characteristics, earthworms can be divided into three main ecological groups—epigeic, endogeic and anecic species (Bouché, 1977). The differences in the sensitivities of the species from different ecological groups to various toxicants have been investigated, but only by conducting laboratory toxicity tests (Ma and Bodt, 1993; Spurgeon and Hopkin 1996; Langdon et al., 2005; Velki and Hackenberger, 2012, 2013c). Due to the detected differences, when using earthworms as model organisms, the differences in species sensitivities must be considered.

Standardized toxicity tests focus on assessing the effects of toxicants on single species and are conducted under controlled laboratory conditions that are considerably different from the conditions of a realistic environment. The response of earthworms to pollutants is dependent on the conditions under which they are exposed and the differences between laboratory and environmental conditions can significantly affect the results of the research (Spurgeon et al., 2005b). In laboratory investigations, it is possible to achieve the conditions of the experiment close to environmental conditions by using microcosmic systems. A terrestrial microcosm can be defined as a soil unit consisting of treated (collected and sieved) soil or intact soil cores with several species introduced in order to assess the impacts of the pollutants at different levels of the biological organization (Burrows and Edwards, 2002). The utilization of a microcosm can reduce the possibility of an inaccurate estimation of the adverse effects of pollutants, and in the present research a microcosm was used for assessing the effects of insecticides on earthworm species belonging to different ecological categories.

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* Corresponding author. Fax: +385 31 399 921.
E-mail addresses: mirna.velki@gmail.com (M. Velki), hack@biologija.unios.hr (B.K. Hackenberger), zloncaric@biologija.unios.hr (Ž. Lončarić), davorka@biologija.unios.hr (D.K. Hackenberger).

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Eisenia andrei (Bouché, 1972) is a compost epigeic species commonly used in laboratory experiments but it is not significantly represented in the environment. In this study *E. andrei* was used in order to enable the sensitivity comparison and comparison of results from previous investigations (Velki and Hackenberger, 2013a, 2013b). *Lumbricus rubellus* (Hoffmeister, 1843), a widely distributed epigeic species, and *Octolasion lacteum* (Örley, 1885), an endogeic species, were chosen based on their ecological category and the previous research conducted on investigated insecticides (Velki and Hackenberger, 2012, 2013c). *Lumbricus terrestris* (Linnaeus, 1758) has been used as a model species for assessing the effects of different pollutants (e.g. Fitzpatric et al., 1992; Vejares et al., 2010; Calisi et al., 2013), and in this study was chosen as a representative of the anecic category. In a majority of the previous studies, only the epigeic *Eisenia* was used due to easy laboratory breeding and maintenance; however, due to the prevalence of endogeic and anecic species in the environment (Edwards and Bohlen, 1996) it is necessary to include species from these categories in the (eco)toxicity investigations.

In this research, the effects of three commonly used insecticides were investigated—organophosphates dimethoate, pirimiphos-methyl and pyrethroid deltamethrin. The toxicological effects of organophosphates are based on the inhibition of the enzyme acetylcholinesterase that leads to the accumulation of acetylcholine, excessive stimulation of cholinergic receptors and disruption of neural activity in the organism (Stenersen, 2004). Deltamethrin is considered the most powerful synthetic pyrethroid (Bradbury and Coats, 1989) and the mechanism of the action of pyrethroids is based on the excitation of the sodium and potassium channels of the neurons and the delayed closure of channels during the phase of depolarization (Stenersen, 2004). The effects of these insecticides were evaluated by measuring the molecular biomarkers (activities of acetylcholinesterase, carboxylesterase, catalase, glutathione S-transferase and glutathione concentration) which may act as a sensitive, early warning indicator of the possible effects at higher levels of biological organization (Spurgeon et al., 2005a) and may provide information on the mode of action of a chemical (Kammenga et al., 2000). Also, the importance of the measurement of the molecular biomarkers is reflected in their possibility to generate insights on the possible harmful effects that cannot be obtained from chemical analysis alone (Depledge and Fossi, 1994) because biomarkers focus on the effects of the bioavailable fraction of and provide the determination of the direct effects of the toxicant on organisms. In previous studies (Velki and Hackenberger, 2012, 2013a, 2013b, 2013c) the effects of dimethoate, pirimiphos-methyl and deltamethrin on the molecular biomarkers in *E. andrei*, *O. lacteum* and *L. rubellus* earthworms were investigated, but only under laboratory conditions using a standard filter paper contact test and an artificial soil test (OECD, 1984).

As pointed out by Pelosi et al. (2013), there are knowledge gaps in the investigation of the effects of pesticides on earthworms and one of the main conclusions of their review is that, in a European context, there is a lack of studies based (1) on European species and using pesticides still authorized in Europe, (2) on species that are actually found in the environment and not only epigeic Eisenia species and (3) on realistic conditions in terms of soil, pesticide dose and experimental duration. The present study addresses all of these points and the investigation of pesticide effects is conducted by the simultaneous exposure of earthworm species belonging to different ecological categories using the soil microcosm. When comparing a new method with an established one, the information on the required sample size is crucial to assess whether the new measurement provides substantial advantages compared to the established method. The number of samples requisite for certain experimental design depends on the variability of the response being measured and the effect size to be detected. If a new method requires a higher number of samples, the relevance of the obtained results must justify a larger sample size. Therefore, one of the issues that will be addressed in this research is the difference in the required sample size in a microcosm vs. a standardized toxicity test. The main research objectives of the present study were to investigate the effects of environmentally relevant concentrations of three insecticides on earthworms under environmentally relevant conditions; to compare the biomarker responses of different earthworm species in order to determine the species' susceptibility to a particular insecticide; and to compare biomarker responses recorded after pesticide exposure in a microcosm with responses recorded in previous studies after pesticide exposure using standardized toxicity assays.

2. Materials and methods

2.1. Earthworms and soil sampling

The adult *E. andrei* earthworms were obtained from a culture maintained in our laboratory. Specimens of *O. lacteum*, *L. rubellus* and *L. terrestris* were collected in spring 2012 by hand sorting from an uncontaminated grove. The earthworms were all adults with well-developed clitellae—0.24 ± 0.05 g for *E. andrei*, 0.39 ± 0.09 g for *O. lacteum*, 0.41 ± 0.09 g for *L. rubellus*, 1.97 ± 0.48 g for *L. terrestris* (mean ± SD of mass of earthworms after voiding the gut content). Identification and taxonomic assignment of adult earthworms was performed according to Csuzdi and Zicsi (2003). Soil for the microcosm was collected from an uncontaminated site in Baranja (Osijek-Baranja County) situated in the north-eastern part of the Republic of Croatia. The sampled soil was sterilized, homogenized, sieved and used for the microcosm. The soil in the microcosmic system was slightly alkaline (pH 7.5), sandy clay (50.5 percent of clay, 8 percent of silt, and 41.5 percent of sand), the organic matter content was 3.8 percent and the water holding capacity was 42 percent.

2.2. Chemicals

All reagents used were of analytical grade. 5,5'dithiobis-2 nitrobenzoic acid (DTNB), acetylthiocholine iodide (AcSChI), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), 4-nitrophenyl acetate, bovine serum albumin (BSA) and rhodamine B (RB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following commercial preparations of pesticides were used—Chromgor 40 (Chromos Agro d.d., Zagreb) as dimethoate preparation (400 g/L of dimethoate; 18 percent Solvesso 150 which is mostly composed of a heavy aromatic solvent naphtha); Actellic 50 EC (Syngenta Agro d.o.o., Zagreb) as pirimiphos-methyl preparation (500 g/L of pirimiphos-methyl; <500 g/L light aromatic solvent naphtha; <100 g/L of calcium alkyl benzene sulfonate); and Rotor 1,25 EC (Chromos Agro d.d., Zagreb) as deltamethrin preparation (12.5 g/L of deltamethrin; 90 percent Solvesso 100 which is mostly composed of a light aromatic solvent naphtha).

2.3. Microcosm

The microcosmic system (Supplementary Fig. S1), comprised of cylindrical plastic drainpipes 1 m in height and 30 cm in diameter. The bottoms were filled with a layer of bricks and gravel which served as a drainage system. On top of the gravel was a thin sponge (30 cm in diameter, 1 cm thick) which allowed the passage of water and prevented the passage of earthworms. Approximately 45 kg of soil was placed in the pipes (soil column of 65 cm). Additionally, horse manure was dried, sterilized, ground and mixed with the soil in order to provide food for the earthworms. *O. lacteum*, *L. rubellus* and *L. terrestris* earthworms were placed together (25 earthworms of each species). Due to a possible negative impact on the other species in the microcosm (see Section 4.1), *E. andrei* were placed separately (75 earthworms). The soil placed in the microcosm was allowed to stabilize for 2 weeks and afterwards the earthworms were added and acclimatized for 10 weeks. After this period, the pesticides were applied to surface of those microcosmic systems and the exposure started. In order to achieve conditions close to environmental conditions, the average precipitation for the spring/summer period was calculated (65 mm/month) and the microcosm was watered accordingly. The microcosmic systems were placed under a natural long-day photoperiod with an average air temperature of 22 °C (18–26 °C) during the experiment and the variation in day and night temperature was maintained in order to have conditions as close to the environmental conditions as possible.