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Original article

Metallothionein expression and Neutral Red uptake as biomarkers of metal exposure and effect in *Eisenia fetida* and *Lumbricus terrestris* exposed to Cd

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Abstract

In this study we compared short-term, sub-lethal responses of two different earthworm species, *Eisenia fetida* and *Lumbricus terrestris*, exposed to Cd (100 mg/kg) under laboratory conditions. Biological responses at the cellular and molecular genetic levels of organisation were measured. First, the Neutral Red uptake test was performed on extruded coelomocytes. Observations showed a significant reduction in cell membrane integrity in *E. fetida* exposed to Cd, whilst *L. terrestris* membranes appeared to be unaffected. Second, metallothionein 2 (MT2) gene expression levels were measured by Q-RT-PCR. Observations showed a significant up-regulation of the Cd-inducible metalloprotein in both species. Transcriptome measurements indicated that basal MT gene expression levels were higher in *E. fetida* than in *L. terrestris*, but Cd-induced MT2 up-regulation was approximately similar in samples from the two species. In conclusion, the susceptibility of coelomocyte membranes to Cd-evoked toxicosis is not correlated with Cd-induced MT2 expression levels.

Keywords: Neutral Red uptake; Metallothionein; Earthworms; Cadmium; Q-RT-PCR

1. Introduction

Terrestrial pollution by toxic metals is widespread and, in most urban regions, occurs at low or moderately elevated levels. Reliable soil health criteria must be established, supported by robust, sensitive, and meaningful site-specific assays of pollution intensity and effects. Assessment of metal availability is often based on total or extractable soil concentrations [23,28]; but, more recently, accumulated metal burdens [29] and the cellular fractionation of metals [36] in ecologically important sentinel organisms, such as earthworms, have been advocated. Evaluation of soil health using earthworms can be done by measuring changes in demographically-related whole-organism parameters, such as mortality, growth or reproduction; alternatively, earthworm performance may be evaluated with a biomarker approach [31]. The latter approach gives information about

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changes occurring at low levels of biological organization, with the possibility of predicting consequential, ecological level changes, at early states of pollution and at sub-lethal concentrations of contamination.

Techniques involving *in vitro* tests are increasingly used as alternatives to whole animal toxicity tests due to their reduced use of experimental animals, low cost, and rapidity [27]. The earthworm immune system consists of different lineages of coelomocytes. The collection of viable earthworm coelomocytes collection is easy and clean, making these fluid-suspended cells suitable for developing *in vitro* tests [10]. It has been shown that different pollutants (organic and inorganic residues) can perturb integrity and functions of earthworm coelomocytes, and that these responses can be used as biomarkers of sub-lethal, chemical-induced, stress [31]. Neutral Red uptake (NRU) assay has been used previously for the identification of vital cells in cultures [7]. This assay quantifies the number of viable, uninjured cells after their exposure to toxicants; it is based on the uptake (endocytic activity and passive diffusion) and subsequent lysosomal accumulation of the supravital dye, neutral red, by living, but not by dead cells. Therefore, it can be used as an indicator of intact cell membrane integrity. It has been used in marine invertebrates to estimate changes in the endocytic capability of haemocytes in response to environmental pollution [15,16,27]. Recently the test has been used on earthworm (*Allolobophora chlorotica*) coelomocytes to assess the toxic effects of Zn, Pb, Cd and Cu [17], and in several earthworm species (*A. chlorotica, Lumbricus terrestris, Dendrobaena veneta* and *Eisenia fetida*) to study responses to shock [21].

High-throughput technologies, such as quantifying gene expression by Q-RT-PCR, can be used to develop new biomarkers for ecotoxicological studies [13,14,33]. There are a certain amount of proteins related to metal exposure and stress situations which can be used as biomarkers of exposure. Metallothioneins are small cysteine-rich, metal-inducible, proteins that are amongst the most intensively studied due to their function in non-essential metal homeostasis, sequestration and detoxification; and they can be found in the gut epithelium, typhlosole, coelomocytes and nephridia of earthworms [1,5,35].

The main aim of this study was to compare the responses of two ecophysiologically contrasting earthworm species (epigeic *E. fetida* and anecic *L. terrestris*) under short term Cd-exposure by means of cell and molecular biology approaches in order to develop biomarkers able to be used in soil health assessment. A subsidiary aim was to compare the efficacies of a cellular biomarker (neutral red uptake by extruded coelomocytes) and a molecular genetic biomarker (Q-RT-PCR of a metallothionein isoform, w-MT2, implicated in Cd detoxification in earthworms).

2. Materials and methods

2.1. Earthworms

E. fetida were obtained from a commercial dealer (Manchaverde SL, Ciudad Real, Spain), while *L. terrestris* were collected from an unpolluted area. Prior to the experiment, earthworms were kept in tanks at 19 °C and 12:12 h light/dark cycle, and horse manure was added as food as required. The earthworms used in the experiment were all healthy, clitellated, and of similar size (300–600 mg fresh weight for *E. fetida,* 5–6 g fresh weight for *L. terrestris*).

2.2. Experimental design

The growth medium was a clean and well characterised rural soil (Table 1). Soil samples were artificially contaminated with $CdCl_2$ dissolved in distilled water to provide the nominal exposure concentration of 100 mg Cd per kg dry soil (LC50/10 for *E. fetida* in OECD artificial soil [18]. Earthworms, *E. fetida* and *L. terrestris*, were maintained for 3 days in the following experimental conditions: constant light, in 3-L glass containers, 35–40% humidity, at 19 °C; two replicates of each experimental group were carried out.

Table 1
Soil characteristics of the basic substrate used in this experiment

	Basic substrate
pH	6.9
EC	2520 μS/cm
OM	14.35%
N	0.64%
C/N	13
Texture	Loam clay
NO_3	95.3 mg/L
P	>120 mg/L
Na	38 mg/L
K	454 mg/L
Ca	4460 mg/L
Mg	586 mg/L
Cr	29.9 mg/kg
Cd	1.05 mg/kg
Cu	48.3 mg/kg
Ni	10.9 mg/kg
Zn	176 mg/kg
Pb	46.5 mg/kg