Ecological Indicators 60 (2016) 377–384

Contents lists available at ScienceDirect

Ecological Indicators

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

Viability of gut microbes as a complementary earthworm biomarker of metal exposure

Shin Woong Kim, Yooeun Chae, Jin Il Kwak, Youn-Joo An*

Department of Environmental Science, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea

ARTICLE INFO

Article history:
Received 1 January 2015
Received in revised form 11 July 2015
Accepted 13 July 2015

Keywords:
Earthworm
Flow cytometry
Gut microorganisms
Metal toxicity

ABSTRACT

Earthworms are standard species used in soil ecotoxicology to evaluate the adverse effects of soil contaminants. This study proposes the assessment of the viability of earthworm gut microbes as an indicator in a site-specific test of soil toxicity. Using slow centrifugation, the microbial community was extracted from the guts of earthworms that had been exposed to copper (Cu)- or nickel (Ni)-contaminated soil. Microbial cell viability was assessed using calcein acetoxymethyl ester staining and flow cytometric analysis. We confirmed a metal concentration-dependent decrease in the cell viability of the gut microbial community. The general endpoints, including survival, abnormalities, coelomocyte activity, and metal bioaccumulation, showed a metal concentration-dependent response, and were strongly associated with gut microbial viability in the Ni-exposure group. In contrast, the general endpoints in the Cu-exposure group were significantly different from those in the former group, because the soil penetration rate of the earthworms was very low on the Cu-contaminated soil. Our results indicated that the gut microbial community viability assay holds potential for assessing the toxicity of soil to field worms by simply and rapidly monitoring the viability of the earthworm gut microbial community.

1. Introduction

Earthworms are one of the representative soil invertebrates, and play ecologically important roles in the health of soil ecosystems. They modify the physical structure of the soil by improving the porosity and by producing new aggregates. Earthworms ingest a large amount of soil, organic matter, and surface litter, and this feeding activity raises the decomposition rate and nutrient cycling (Edwards and Bohlen, 1966; Horn et al., 2003; Zhang et al., 2000). These activities commence in the gut of the worm (Gómez-Brandón et al., 2011). Microbiological investigations have shown that earthworms and their microbial communities (bacteria and fungi) have strong interactions, including competition, mutualism, predation, and facilitation (Sampedro and Domínguez, 2008). Earthworm (epigeic, anecic, and endogeic species) casts provide some physicochemical and biological benefits to soil (Brown et al., 2000). This material is a nutrient-rich source and function as a hotspot for microbial proliferation (Aira et al., 2010). The casts that are returned to the soil are therefore enriched with nutrients and microorganisms, which benefit soil health (Edwards and Bohlen, 1966; Sen and Chandra, 2009; Vivas et al., 2009).

Earthworms are also standard species used in soil ecotoxicology for evaluating the adverse effects of soil contaminants (Van Straalen and van Gestel, 1998), which focus on measuring mortality, behavior, pathological symptoms, body weight change, and reproductive activity by established methods (ISO, 2008, 2012a,b; OECD, 2004; USEPA, 2012). Previous studies on earthworms have investigated the effects of toxicants on the lysosomal stability of coelomocytes by assessing neutral red retention (Svendsen et al., 2007), phagocytosis (Fuller-Espie et al., 2010), DNA damage (Manerikar et al., 2008; Li et al., 2009), and enzymatic responses (Li et al., 2009). These biomarkers are very useful for evaluating the toxicity of chemicals, and facilitate evaluation of the risks of contaminant exposure (Fernandez et al., 2005).

In site-specific soil assessments, toxicity can be evaluated by exposing the organisms to specific contaminated samples, and various species are commonly used as relevant indicators. Avoidance tests of earthworms or springtails have been suggested as rapid bioassays for evaluating contaminated soil samples (Antunes et al., 2008; Loureiro et al., 2005; Natal-Da-Luz et al., 2008). Microbial bioassays (soil respiration, contact test, enzymatic activities), bait-lamina tests, Microtox®, and elutriate assays have been employed for screening site-specific toxicity (Abbondanzi et al., 2003; Brohon et al., 2011; Robidoux et al., 2004). Most of these bioassays have been implemented as laboratory-scale techniques and screening

* Corresponding author. Tel.: +82 2 2049 6090; fax: +82 2 2201 6295.
E-mail address: anyjoo@konkuk.ac.kr (Y.-J. An).

http://dx.doi.org/10.1016/j.ecolind.2015.07.010
1470-160X/

tools, and some assays were adapted from aquatic ecotoxicological methods.

However, there is a need for the development of toxicity assays that can assess the site-specific toxicity to a field acceptor. Some studies have shown field applicability and potential use of bioassays and biomarkers by using genotoxic responses, bioaccumulation or tissue residues (Marinussen et al., 1997; Ma, 2005), metabolomic responses (Åslund et al., 2012), and coelomocyte contents in earthworm species (Plytycz et al., 2009; Lourenço et al., 2011). However, these studies still does produce incontrovertible results and have limited applications, as changes are unmeasureable in some cases (Plytycz et al., 2009), the relevance of the results to other endpoints are uncertain (Åslund et al., 2012), and some metabolic properties are unknown (Lourenço et al., 2011).

Here, we suggest a gut microbial community viability assay as a simple and rapid bio-technique for assessing soil toxicity in a site-specific manner. The aims of this study were: (1) to assess the response of the gut microbial community in earthworms acutely exposed to metal-contaminated artificial soils, using flow cytometry, and (2) to compare flow cytometry outcomes with common toxicity endpoints, such as survival, coelomocyte viability, or metal bioaccumulation. Fresh gut contents were extracted after exposure of worms to metal-contaminated soils, and the cell viability of microorganisms in the gut was measured using flow cytometric approaches with calcein acetoxymethyl ester (calcein-AM) staining. We propose that this bioassay can be applied to field-worms, and that this simple and rapid monitoring of gut microbes in earthworms holds strong potential as a site-specific indicator of soil toxicity.

2. Materials and methods

2.1. Test organism and materials

The test species used was *Eisenia andrei*, which is recommended as a standard test species by the OECD testing guideline (OECD, 2004). Earthworms were maintained in modified OECD soil containing extra peat moss with cereals as food source (20 ± 1 °C in darkness). Before the test, the adult earthworms (300–600 mg) were maintained on moist filter paper for 3 h at 20 ± 1 °C in darkness to remove all gut contents. Copper ($CuCl_2 \cdot 2H_2O > 99\%$) and nickel ($NiCl_2$, 98%) were chosen as test chemicals, and calcein-AM was selected for evaluation of cell viability of the microorganisms. All materials were obtained from Sigma Chemical Co., Inc. (St. Louis, MO, USA).

2.2. Acute toxicity assay

Acute toxicity assays were performed using modified OECD Testing Guideline (TG) No. 207. Landwirtschaftliche Untersuchungs and Forschungsanstalt (LUFA 2.2; Speyer) was used as test soil. The pH, organic matter, organic carbon, and the texture of LUFA 2.2 were 5.5 ± 0.2, 3.82%, 1.77%, and loamy sand, respectively. Ten grams of LUFA soil was placed in a flat-bottom vial (ID: 25 mm, height: 50 mm, volume: 20 mL) with ten replicates, and the test solution was spiked into the soil with a water content of 35% (v/w). Cu and Ni concentrations of 0, 100, 200, 300, 400, and 500 mg/kg dry soil were prepared. One *E. andrei* worm was placed into each test vial, and a sili stopper was used to prevent avoidance. Exposure conditions were 20 ± 1 °C and darkness. After 3, 5, and 7 days, the survival rate, abnormalities (mucous secretion, bleeding, swelling, thinning, and/or fragmentation), and soil penetration rate were observed. All data were represented as the percentile compared to the control group (no metal exposure).

2.3. In vivo *cytotoxicity assay using earthworm coelomocytes*

After the acute toxicity assay, the survivors were collected for *in vivo* cytotoxicity assays. Coelomic fluids were directly extracted from the worms by placing a hypodermic syringe containing 0.1 mL Lumbricus balanced salt solution (LBSS: 1.5 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 4.3 mM $NaHCO_3$, and 3.8 mM $CaCO_3$) into the clitellum of the worm (Sauvé et al., 2002; Svendsen et al., 1996).

Calcein-AM was used as a fluorescent dye for evaluating the esterase activity of earthworm coelomocytes. Calcein-AM was diluted using LBSS from a 500-μM stock solution in DMSO, and was mixed with cell suspensions (coelomic fluids) as final concentration of 5 μM of calcein-AM in 1 mL of LBSS. Samples were incubated for 60 min at 37 °C. Cell suspensions of *E. andrei* after calcein-AM staining were centrifuged for 1 min at $2450 \times g$. The resultant pellets of coelomocytes were resuspended in 1 mL of LBSS.

We used flow cytometry (FACScalibur, BD Biosciences, San Jose, CA, USA) to calculate the concentration of the green fluorescent calcein. Cell events were determined as 20,000 events, and the intensity values were analyzed with FL-1 (500–560 nm band pass filter, green fluorescence) after excitation at 488 nm. The results were analyzed with Cell Quest Pro software (BD Biosciences, San Jose, CA, USA), and the esterase activity of the coelomocytes was expressed as the geomean of the calcein intensity.

2.4. Bioaccumulation analysis

Total concentrations of Cu and Ni were measured in three earthworm survivors of the acute toxicity assay, which were washed in distilled water, and maintained on moist filter paper to remove any soil and gut contents. After 24 h, whole worms were dried at 100 °C, and 3 mL of nitric acid (70%) was added to the worms in digestion beakers. Samples were heated using 24-well hot plates (THB-1024 HOT Block Digester, Tekton, Korea) at 121 °C overnight. The total concentration of Cu and Ni in the earthworm was measured using ICP-AES (JY-138-UL-TRACE, Jobin-Yvon, France) after acid digestion of the sample.

2.5. Assessment of cell viability of the soil microbial community

Soil microorganisms were extracted using the rotating-low centrifugation method described by Lindahl and Bakken (1995) with minor modifications. Soil samples from the survivors of the acute toxicity assay were mixed using a spatula. Of this soil, 0.5 g was placed into 15-mL test tubes, and 5 mL phosphate-buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2PO_4, and 0.24 g/L KH_2PO_4) was added, in six replicates. Samples were then vortexed for 1 min, and centrifuged at low speed ($650 \times g$) to extract the soil microbial community from the soil particles. The extracted microbial community was stained with calcein-AM in PBS solution, as described above. Flow cytometric conditions involved monitoring 10,000 cell events in the FL-1 channel. A time-dependent assay was conducted to observe the viability of the microbial community in the soil present in the earthworm. The soil was prepared using the same conditions as in the acute assay, but without metal chemicals, and the soil not obtained from an earthworm was used as control. The soil microbial community was extracted every day for 7 days as described above, and was analyzed by flow cytometry with calcein-AM staining.

2.6. Gut microbial community viability assay

Gut microbial communities were separated from the fresh gut content of the metal-exposed worms. Six worms were washed using distilled water after the acute toxicity assay, and the