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Toxic effects of PCDD/Fs mixtures on *Eisenia andrei* earthworms

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ABSTRACT

The earthworms *Eisenia andrei* were used to study the toxicity of PCDD/Fs mixtures to earthworms during 28 day of exposure. The experiments were performed on artificial soils contaminated with dioxins at levels of C1 (0.1 ng 2, 3, 7, 8-TCDD/g soil), C2 (1 ng 2, 3, 7, 8-TCDD/g soil) and C3 (1.5 ng 2, 3, 7, 8-TCDD/g soil). Effects of PCDD/Fs on survival, growth rate and immune responses; phagocytosis and NK-like cell activity, were determined. No mortality was observed at the lowest concentration (C1), while mortalities of 10 and 100% were noted at the highest concentrations tested C2 and C3, respectively. A significant reduction in growth rate was obtained at C2 and no effects at C1. Additionally, an inhibition of phagocytic activity and efficiency was observed at higher concentrations. In contrast, an enhancement of NK-like cell activity was shown at lower concentrations. Based on our results, we hypothesize that the PCDD/Fs mixtures tested at levels equal or higher to C2 (1 ng 2378-TCDD/g soil), lead to adverse effects on biotic potential and immune functions in *E. andrei* earthworms.

1. Introduction

In soil ecotoxicological studies, earthworms have been extensively used as bioindicators and test organisms for measuring the lethal and sub lethal effects of chemicals in the soil environment. Earthworms constitute approximately 60–80% of the soil biomass (Rida, 1994) and play an important role in soil formation processes and in maintaining soil structure and fertility. Most of these studies have used the compost worms *E. fetida* or *E. andrei* due to their availability and because they are relatively easy to handle in the laboratory. In addition to their sensitivity to chemicals; the tissues of earthworms can concentrate some insecticides (Cooper and Roch, 2003), trace metals (Homa et al., 2005; Asensio et al., 2007; Lee and Kim, 2008) and persistent organic pollutants (POPs) (Vermeulen et al., 2010). To date, some studies on POPs have investigated the effects of PCBs (Goven et al., 1994a, 1994b; Ville et al., 1995), dioxin-like PCBs (Bu et al., 2010) and PAHs (Komiyama et al., 2003) on earthworms, but few data have been reported on the effects of PCDDs/Fs (Reinecke and Nash, 1984). It is well recognized that PCDDs and PCDFs enter the environment either through unintentional application as by-products of combustion processes and represent one of the most toxic anthropogenic chemicals in the environment given their stability, lipophilicity, bioaccumulation potential and high persistence. In addition, numerous studies have reported that prolonged exposure to TCDD presents adverse health effects, including immunotoxicity, neurotoxicity, hepatotoxicity and carcinogenesis (Wyde et al., 2002; Alaluusua et al., 2004; Baccarelli et al., 2005; Eskenazi et al., 2005; Lin et al., 2007). Moreover, the International Agency for Research on Cancer (IARC) has classified TCDD as a Group 1 carcinogen i.e., a human carcinogen (IARC, 1997; ATSDR, 1998; ATSDR, 2006). Environmental exposure concentrations of 1 ng 2, 3, 7, 8-TCDD/g soil were considered "a level of concern" for causing cancer (Pohl et al., 1995). Different concentrations of dioxins were found in the soil, in the vicinity of industrial area. For example; Rovira et al. (2010) reported values of 0.14–0.46 pg-I-Teq/g in Catalonia (spain), 0.6 to 6.38 pg-I-Teq/g were noted in Hangzhou (china) (Xu et al., 2009) and 0 to 153.23 pg-I-Teq/g were detected in Nationwide in Korea (Kim et al., 2008). Thus, the presence of these toxic contaminants in soils may have direct harmful effects to the terrestrial ecosystem.

In order to assess the ecological risks of contaminated soils with different environmental pollutants, standardized tests have been developed. A guideline for conducting soil toxicity tests has

Abbreviations: PCDDs, Polychlorinated dibenzo-*p*-dioxins; PCDFs, Polychlorinated dibenzofurans; NK, Natural killers; PCBs, Polychlorinated biphenyls; PAHs, Polycyclic aromatic hydrocarbons; RPMI, Roswell Park Memorial Institute medium; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

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been reported by the Organisation for Economic Cooperation and Development (OECD, 1984, 2004) or by the International Standard Organization (ISO, 1993; ISO, 1998, 2004). These toxicity tests are used to predict acute and/or chronic effects of chemicals into the environment (Sanchez-Hernandez, 2006). Usually, the typical endpoints measured are survival, growth and reproduction rate. In addition to these parameters, earthworm performance may be evaluated through a biomarker approach studied at cellular and tissue levels (Scott-Fordsmand and Weeks, 2000). Many biomarkers may be employed but in this study, the focus was on immunological biomarkers; phagocytic activity and NK-like cell activity. Since immune responses represent important host defence mechanisms, their modulation may result in increased incidence of infections that could influence the survival of individuals and their populations (Goven et al., 1994a, 1994b).

Previously, the immune system parameters as phagocytosis and NK-like cell activity have been used as sensitive sub-lethal endpoints to assess toxicity of various chemicals to earthworms. It has been shown that the exposure to mercury, cadmium or zinc was toxic and affected cell viability and phagocytosis (Fournier et al., 2000; Sauvé et al., 2002; Massicotte et al., 2003; Sauvé and Fournier, 2005). In addition, Goven et al. (1994a, 1994b) and Ville et al. (1995) showed that the phagocytic competence is also inhibited by polychlorinated biphenyls (PCBs). Thus, the suppression of the phagocytic activity or reduction of cœlomocyte viability decreases animal resistance to infection (Fournier et al., 2000). It was also demonstrated that PCBs (Suzuki et al., 1995) and PAHs (Patel et al., 2007) exerted immunosuppressive effects on NK-like effector cells. This deficiency in immune functions is considered as a sign of toxic effects of environmental pollutants (Fournier et al., 2000).

The objectives of the present study were to investigate, in the laboratory; whether PCDD/Fs exert toxic effects on the growth, the survival and the immune system of earthworms.

2. Materials and methods

2.1. Reagents

The mixture of PCDDs/Fs in nonane solution was obtained from Wellington laboratories (Ontario, Canada) and stored at 1 °C. Dimethyl sulfoxide 99.5% (DMSO), formaldehyde 37%, and phosphate buffer solution (PBS) were obtained from Sigma–Aldrich (USA). The bovine serum albumin (BSA) was purchased from MP Biomedicals, LLc (Ohio, USA) and sodium azide (NaN_3) from Ficher Chemical, USA. RPMI 1640, containing 25 mM HEPES buffer, L-glutamine and sodium bicarbonate, was supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. K562 (Sigma Aldrich) was cultured in this complete RPMI media. The 3,3′-dioctadecyloxacarbocyanine (DIO) was dissolved in DMSO to yield 3 mM. A stock solution of propidium iodide (PI) of 1 mg/ml in water was prepared. Purified water was obtained from a Milli-Q water purification system.

2.2. Earthworms

The earthworms, *E. andrei* were initially purchased from Carolina Biological Supply (Burlington, NC, USA) and were used to establish the laboratory culture. Prior to the experiment, the animals were maintained in earthworm bedding (Magic Products, Amherst, Jct, WI) at 20 ± 1 °C, 70–80% (w/w) moisture and 16:8 h light/dark cycle, and fed once a week with cereal (Magic Worm Food, Magic Products).

2.3. Soil toxicity tests

The soil toxicity test using *E. andrei* earthworms was carried out, on artificial soil, to determine the relative lethal and sublethal effects of PCDD/Fs-spiked soil samples following exposure by direct contact and by ingestion. The experiments were based on nominal exposure concentrations and thus have a semi quantitative, mostly comparative character and are focused on the biochemical effects of dioxins upon immune system, growth and survival of earthworms, and not on the toxicity values for regulatory purposes.

Table 1
Initial concentrations of PCDD/Fs mixtures in artificial soils.

PCDD/Fs	C1 (ng/g)	C2 (ng/g)	C3 (ng/g)
2378-TCDD	0.1	1	1.5
2378-TCDF	0.1	1	1.5
12378-PeCDD	0.25	2.5	3.75
12378-PeCDF	0.25	2.5	3.75
123678-HxCDD	0.25	2.5	3.75
123678-HxCDF	0.25	2.5	3.75
1234678-HpCDD	0.25	2.5	3.75
1234678-HpCDF	0.25	2.5	3.75
OCDD	0.5	5	7.5
OCDF	0.5	5	7.5

The earthworm exposures were realised in standard conditions following the US EPA and the OECD guidelines (USEPA, 1989a; OECD, 1993). Briefly, the earthworms used in the toxicity tests were healthy adults, presenting clitellum and a body mass ranging from 300 to 600 mg fresh weight. The soil used was an artificial OECD-type soil consisting of 10% (w/w) sphagnum peat, 20% (w/w) kaolinite clay, 70% (w/w) quartz sand, and adjusted to pH 6.5 with $CaCO_3$ (OECD, 1993). The test soil was placed into clean 1-L glass jars at 500 g (dry weight) for each jar. The test mixture of PCDD/Fs was first dissolved in DMSO (final concentration 0.01%) before being spiked into the soil to make final concentrations of C1, C2 and C3, where 2, 3, 7, 8-TCDD concentrations were 0.1, 1 and 1.5 ng/g soil, respectively (Table 1). The PCDD/Fs concentrations were chosen based on "the level of concern" of 2, 3, 7, 8-TCDD (1 ng/g soil) described in the literature (Pohl et al., 1995). Three jars were prepared for each concentration. Negative controls received no PCDD/Fs and were included along the test with controls receiving DMSO (without PCDD/Fs) to test for any effect due to the solvent (vehicle). The soil humidity was then adjusted to 75% of the total water-holding capacity of the soil as described in literature (Robidoux et al., 1999). Before starting the test, the worms were acclimated for 24 h. Then, ten earthworms, individually washed and weighed, were placed in each replica glass jar. The jars were covered with a geotextile membrane and a perforated cap and were maintained at 20 °C with a 16:8 h light/dark cycle. The earthworms were fed weekly with 2 g of dry cereal.

The percentages of survival and growth rates were determined every week. The growth rate was determined using Eq. (1), where W_0 is the mean weight at the beginning of exposure and W_t the mean weight after t days of exposure (Martin, 1986).

$$\text{Relative growth rate} = \ln(W_t/W_0) \times 100 \quad (1)$$

After 28 day of exposure, the live worms were rinsed with deionized water, blotted dry and individually weighed before being placed for 3 h on moist Whatman filter paper in Petri dishes (Furst and Nguyen, 1989) to extract ingested soil.

2.4. Cell extrusion

Five purged earthworms from each jar were used for the measurement of the immunological biomarkers; phagocytosis and NK-like activity. A single worm was inserted into a 15-ml tube containing 3 ml of Lumbricus balanced salt solution (LBSS) composed of 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 $Na_2PO_4 \cdot 7H_2O$, 3.8 mM $CaCl_2$, and 4.3 mM $NaHCO_3$ (Brousseau et al., 1997, 1999). Cœlomocytes were extracted using an electrical extrusion method which consists in submitting the liquid medium to a 6 V current (lantern battery) for 20–30 s using aluminium wires (Massicotte et al., 2003). The worm was then removed from the tube and the solution was gently shaken.

Cell concentration and initial cell viability were determined by diluting 50 μl of cell suspension with 50 μl of 0.4% trypan blue (Sigma Chemical Co. St. Louis, MO). This mixture was placed into an improved Neubauer hemocytometer which was microscopically observed (Sauvé et al., 2002; Massicotte et al., 2003).

2.5. Phagocytosis

The phagocytic activity of cœlomocytes was measured in each worm, based on the protocol of (Brousseau et al., 1999). For each cell suspension, a volume of fluorescent latex beads (Polysciences Inc., Warrington, PA, USA) was added in a bead: cell ratio of 100:1. The beads were previously sonicated for 5 min at room temperature to get rid of doublet and triplet beads. The cells (with beads) were then incubated for 18 h at room temperature. To remove the beads that were not phagocyted, the cell suspension was layered over a 3% bovine serum albumin (BSA) and centrifuged at 150 g, 4 °C and for 8 min. The cells collected in the pellet were then resuspended in 500 μl of hematall (Fisher Scientific, Ottawa, Ont, Canada) containing 0.185% formaldehyde and 0.2% sodium azide. Thus, the phagocytosis was measured by flow cytometry in FL1 (Becton Dickinson, San José, USA) (Brousseau et al., 1999) and for each sample, the fluorescence of 5000 events was recorded. Results were analysed