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Effects of Incubation Time and Organism Density on the Bioaccumulation of Soil-Borne *p,p′*-DDE by the Earthworm, *Eisenia fetida*

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Abstract Laboratory-scale experiments were conducted to assess the influence of incubation time and organism density on bioaccumulation of weathered *p,p′*-dichlorodiphenyldichloroethylene (*p,p′*-DDE) from soil by the earthworm *Eisenia fetida*. Bioaccumulation was measured after 14, 28, 42, and 56 days of exposure. Tissue concentration increased with incubation time and steady state was not reached until at least 42 days. Organism density had no effect on the bioaccumulation of weathered *p,p′*-DDE. Ratios of 10, 20, 40, and 80 earthworms/350 g of soil led to the same tissue concentrations in test organisms. Risk assessments of contaminated soil should account for these experimental variables.

Keywords Bioaccumulation · Bioavailability · Earthworm · DDE

Due to the potential adverse effects of persistent organic pollutants (POPs) such as DDT and DDE, there has been a great deal of research on the factors controlling their biological availability. For example, chemical properties of soils and pollutants as well as compound residence time in soil have been shown to impact bioaccumulation of organic contaminants (Alexander 2000). Biological factors such as species differences and interactions also appear to exert a substantial influence on the uptake of toxic compounds from soil. Recent work conducted in this laboratory suggested that bioaccumulation of *p,p′*-DDE by the earthworm *Eisenia fetida* was an order of magnitude higher than that of *Lumbricus terrestris* and that plant–earthworm interactions led to profound changes in the uptake of the same compound (Kelsey and White 2005).

Pollutant uptake by earthworms is of interest because these organisms contact soil directly and can act as a conduit through which pollutants enter food webs. Moreover, they are the model organisms used in certain standardized tests designed to evaluate the risk of contaminated soil (OECD 1984). This paper reports the effects of two important variables on the bioaccumulation of *p,p′*-DDE by *E. fetida*, the species recommended by the Organization for Economic Cooperation and Development (OECD) for use in the evaluation of toxicity of soil pollutants to earthworms (OECD 1984): incubation time in contaminated soil and organism density. Duration of exposure to contaminants has been shown to affect uptake in a number of studies. Bioaccumulation factors (BAF, the dry-weight ratio of contaminant concentration in tissue to that in soil) for organic pollutants typically increase and then reach a steady state in short-term studies involving earthworms (Johnson et al. 2002; Matscheko et al. 2002; Sun et al. 2005; Bogan et al. 2005; Hallgren et al. 2006). Although the OECD guidelines for toxicity testing recommend 14 days of exposure, intervals in these studies ranged from 7 to 28 days. Longer-term exposure (i.e., greater than 28 days) are rare. Earthworm density affects worm activities such as growth, feeding, and reproduction (Johnson et al. 2002; Klok 2007) and may influence the uptake of organic pollutants (Johnson et al. 2002). The ratio of earthworm-biomass-to-soil used in published studies varies widely and has the potential to influence the interpretation of data. The objectives of the current study were to systematically examine the effect of exposure time and organism density on the bioaccumulation of the *p,p′*-DDE by *E. fetida*. To our knowledge, such a study has not been conducted previously. Results from this work will

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improve our ability to interpret bioaccumulation data used to assess soil toxicity and to predict the exposure and risk of organic pollutants at contaminated sites.

Materials and Methods

A sandy loam (1.4% organic carbon and pH 6.7) was obtained from the Connecticut Agricultural Experiment Station's Lockwood Farm (Hamden, CT, USA) and stored in covered plastic bins at 22 ± 2°C. Residues of *p,p′*-DDE are present in this soil due to historical applications of DDT to the farm. Prior to experiments, the soil was air dried and hand sieved to 2.0 mm. To determine *p,p′*-DDE contamination levels, 3-g samples of the soil were added to 40-mL amber vials and extracted with 15 mL of *n*-hexanes. One microgram of *o,p′*-DDE (for all forms of DDE, ChemService, West Chester, PA, USA) was added to each vial as an internal standard. The vials were sealed with caps and placed in an oven at 65°C for 5.5 h. Following heating, samples were cooled for 10 min, and a 1-mL aliquot of the supernatant from each was taken and filtered with a glass microfiber filter (0.2 μm, Laboratory Science Inc., Sparks, NV, USA) prior to analysis (see below). The soil used for the contact time experiment contained 410.3 ± 19.6 ng *p,p′*-DDE/g of dry soil (mean and standard deviation of four replicates). Soil containing 248.2 ± 14.4 ng *p,p′*-DDE/g of dry soil (mean and standard deviation of eight replicates) was used for experiments designed to assess the effects of density. The soils were collected from adjacent plots at the Lockwood Farm and were identical except for this difference in *p,p′*-DDE concentration. The soil extraction method used in these experiments was validated previously through comparison with microwave assisted extraction (MAE) of *p,p′*-DDE-contaminated soil (White 2002).

Experiments to test the effect of the two variables were conducted separately (i.e., not concurrently). In all experiments, 350 g of the appropriate dried, sieved soil were added to 600-mL beakers (due to low soil availability, higher organism densities were used than are recommended by OECD guidelines). Water was then added to bring the soils to 20% moisture content (by weight). Mature earthworms (*E. fetida*, obtained from Carolina Biological Supply, Burlington, NC, USA) were washed with tap water and added to the tops of the soils (in the beakers). An average earthworm had a mass of 0.32 g (wet weight). The organisms burrowed into the soil on their own. In experiments designed to assess the effect of incubation time, 20 worms were added to each of 3 replicate beakers. For the density experiments, 10, 20, 40, or 80 earthworms were added to beakers. The number of beakers was 8, 4, 2, and 1 for the 10-, 20-, 40-, and 80-worm treatments, respectively. In all cases, after earthworms were added to soils, the beakers were covered with Al foil (small holes were cut in the foil for aeration) and incubated in the dark at 22 ± 2°C. In the incubation time experiment, organisms remained in soil for 14, 28, 42, or 56 days. In the density experiment, earthworms remained in the soil for 14 days. After the appropriate incubation period, worms were removed from the soil, washed with tap water, and transferred to moistened filter paper (on petri plates) for depuration. All worms survived the experimental period in each experiment and appeared in good health when removed from the soil. Plates were maintained in the dark at 22 ± 2°C. After 24 h of depuration, earthworms from replicate plates were pooled and washed with tap water. The worms were divided into approximately equal portions by mass and transferred to 30-mL vials. The number of replicate portions for the incubation time and density experiments was 6 and 7 per treatment, respectively. Vials were then placed in a freezer (18°C) for approximately 24 h. The earthworm tissues were extracted in 10 mL of hexanes (with 1 μg of *o,p′*-DDE added to each vial as an internal standard) as described above for the soils. Following heating, samples were cooled for 10 min, and a 1-mL aliquot of the supernatant from each was taken and filtered with a glass microfiber filter (0.2 μm, Laboratory Science Inc., Sparks, NV, USA) prior to analysis (described below).

The *p,p′*-DDE content in soil and earthworm extracts was determined using a Hewlet-Packard 5890 gas chromatograph (GC) with a 30 m × 0.32 mm × 0.25 μm HP-5 column, and a ^{63}Ni electron capture device (ECD) (all Agilent Technologies, Inc., Santa Clara, CA, USA). The GC program used was 165°C initial temperature ramped at 10°C/min to 225°C then ramped at 5°C/min to 250°C with a hold time of 0.8 min. The temperature was then ramped at 25°C/min to 280°C with a hold time of 3 min. The total run time was 16 min. A 3-μL splitless injection was used, and the injection port was maintained at 250°C. The carrier gas was H_2 and the make-up gas was N_2 at 60 mL/min. The ECD was maintained at 325°C. Retention times of *o,p′*-DDE and *p,p′*-DDE were 6.17 and 6.74 min, respectively. Crystalline *p,p′*-DDE was added to *n*-hexanes for soil and worm standards at 1,000 ng/mL. The *p,p′*-DDE standard solutions were then diluted to prepare calibration standards of 10, 25, 50, 150, and 250 ng/mL. An internal standard of *o,p′*-DDE at 100 ng/mL was then added to each standard.

Mean values of reported *p,p′*-DDE concentrations from replicate samples as expressed on a dry-weight basis of biomass or soil were used in determining statistical significance. A bioaccumulation factor (BAF) was also calculated for each treatment. Statistical differences among replicates were determined by a Kruskal–Wallis one-way analysis of variances followed by a Tukey multiple comparison analysis ($p < 0.05$).

Results and Discussion

Experimental design affected the uptake of *p,p′*-DDE by *E. fetida* and should be considered when data related to the risk of contaminated soil are interpreted. Bioaccumulation of the compound appears to be controlled by incubation time but organism density, at least in the range tested, has no influence on pollutant uptake.

Bioaccumulation of weathered *p,p′*-DDE by *E. fetida* was dependent on the length of time the organisms remained in contaminated soil. As is reported in Table 1, tissue concentration and BAF increased by a factor of 4 during the 56-day incubation period. The data suggest a progressive change in uptake between 14 and 56 days, although, as shown in Table 1, not all of the differences are statistically significant ($p < 0.05$).

Studies designed to assess the uptake of hydrophobic organic pollutants by earthworms employ different methods to collect data. The OECD guidelines recommend a 14-day exposure when earthworm toxicity is of interest (OECD 1984), yet incubation times reported in the literature typically range from 8 to 28 days. For example, Morrison et al. (2000) used an 8-day exposure and Tang et al. (1999) a 10-day exposure to assess the uptake of DDT, DDE, and DDD by *E. fetida*, van Gestel and Ma (1988) determined earthworm bioaccumulation of chlorophenols after a 14-day incubation period, and Johnson et al. (2002), exposed earthworms to polyaromatic hydrocarbons (PAHs), for 28 days. Both Sun et al. (2005) and Matscheko et al. (2002) concluded that a steady-state tissue concentration had been reached after *E. fetida* was in contaminated soil for 19 days, whereas Hallgren et al. (2006), assumed steady state after *E. fetida* had been exposed to soil containing PCBs for 10 days. Data from the current study indicate that steady state had not been reached after 28 days, and that tissue concentrations of *p,p′*-DDE in *E. fetida* continued to rise until at least 42 days in soil. Although the numbers are not statistically different ($p < 0.05$), the data suggest increases up to 56 days are possible. It is not surprising that different soils, chemical compounds, and organisms could require different lengths of exposure to reach steady state. However, the concentration of pollutants in earthworm tissues determined after short-term (i.e., 10–20 days) incubations might not accurately reflect those found after 56 days or more in soil. Additional research should be conducted to explore the kinetics of uptake and elimination of hydrophobic pollutants by earthworms.

Organism density, unlike incubation time, appeared to exert no influence on the bioaccumulation of weathered *p,p′*-DDE. As is shown in Table 2, neither tissue concentrations of the compound nor BAF values differed appreciably among the four treatments. No statistically significant changes were seen as organism density increased from ten individuals/sample (109.4 g soil/g of biomass) to 80 individuals/sample (13.7 g soil/g tissue).

Like incubation time, earthworm density varies considerably in studies of earthworm accumulation of soil pollutants. The OECD guidelines suggest that ten earthworms should be incubated in 750 g of test soil (OECD 1984) for acute toxicity studies. In the studies cited above that used *E. fetida*, densities ranged from 1.25 (Tang et al. 1999) to 300 g of soil/individual (Johnson et al. 2002), with Hallgren et al. (2006), Morrison et al. (2000), and van Gestel and Ma (1988) using 4, 10, and 50 g of soil/individual, respectively. Interestingly, and consistent with the findings from the current study, although Tang et al. (1999) and Morrison et al. (2000) used rather dissimilar earthworm densities in the same contaminated soil in their respective studies, they reported very similar bioaccumulation factors (0.63 and 0.57, respectively). It should be noted that the uptake of weathered *p,p′*-DDE for the current

Table 1 Effect of incubation time on the bioaccumulation of *p,p′*-DDE by *E. fetida*

Incubation time (days)	Tissue concentration (ng/g of tissue)	BAF[a]
14	2115 ± 858.3A[b,c]	5.1 ± 2.1A[b,c]
28	3704 ± 1070A	9.0 ± 2.6A
42	7250 ± 623.3B	17.7 ± 1.5B
56	8413 ± 1316B	20.5 ± 3.2B

[a] Bioaccumulation factor (BAF) = tissue concentration/soil concentration

[b] Mean ± SD of replicate samples

[c] Within columns, values followed by different letters are significantly different ($p < 0.05$), Kruskal–Wallis one-way analysis of variances followed by a Tukey multiple comparison analysis

Table 2 Organism density and bioaccumulation of *p,p′*-DDE by *E. fetida*

Density (worms/sample)[a]	Tissue concn. (ng/g tissue)	BAF[b]
10	393.3 ± 101[c,d]	1.6 ± 0.4[c,d]
20	402.0 ± 78.7	1.6 ± 0.3
40	428.07 ± 125.6	1.8 ± 0.5
80	366.86 ± 115.1	1.5 ± 0.5

[a] Number of worms in each 350-g soil sample. Mass of an average worm = 0.32 g

[b] Bioaccumulation factor (BAF) = tissue concentration/soil concentration

[c] Mean ± SD of replicate samples

[d] None of the differences among the tissue concentrations or BAF values are significantly different ($p < 0.05$), Kruskal–Wallis one-way analysis of variances followed by a Tukey multiple comparison analysis

experiment was slightly lower than that reported above in the description of the effect of incubation time on the uptake (for 14-day exposures). This discrepancy is not unexpected as the experiments were conducted at different times and used different populations of earthworms. Although the reason for the difference is unknown, slight changes in earthworm maturity and biological variability are the likely explanations.

The fact that density had no influence over bioaccumulation is somewhat surprising in light of what is known about the effect of density on some earthworm species. For example, Postma-Blaauw et al. (2006) noted that soil bacterial communities and nitrogen mineralization depended on the density of *Lumbricus rubellus*, *Aporrectodea caliginosa*, and *L. terrestris*. Klok (2007) described decreases in earthworm growth, maturation, and cocoon production with increased density of *L. rubellus*. There are few references that directly address the effect of organism density on bioaccumulation of hydrophobic organic pollutants, however. Lahr et al. (2008) did observe that the effect of zinc on ecosystem functioning was influenced by the density of *L. rubellus*. Additionally, Johnson et al. (2002) predicted that higher density would be expected to cause lower rates of uptake of PAHs from soil. Much more work needs to be conducted to determine the effect of earthworm density on the bioaccumulation of hydrophobic organic pollutants. Differences and interactions among species as well as properties of soils and pollutants are some of the variables that likely influence density-dependent bioaccumulation and should be examined in future studies.

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