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Short Communication

AVOIDANCE, WEIGHT LOSS, AND COCOON PRODUCTION ASSESSMENT FOR *EISENIA FETIDA* EXPOSED TO C_{60} IN SOIL

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Abstract—*Eisenia fetida* was used as a model terrestrial organism to assess the potential ecotoxicity of molecular pristine C_{60} in soil. Reproduction (assessed by counting cocoon numbers) was hindered only at very high C_{60} concentrations (5% by weight), and C_{60} (up to 1%) was not avoided and did not hinder earthworm growth. This suggests that *E. fetida* is unlikely to experience acute toxicity as a result of C_{60} occurrence in soil. Whether sublethal toxicity may decrease earthworm populations that are chronically exposed to C_{60} at lower concentrations remains to be determined. Environ. Toxicol. Chem. 2011;30:2542–2545. © 2011 SETAC

Keywords—Earthworm   Avoidance test   Reproduction   Nanoparticle   Ecotoxicity

INTRODUCTION

As nanotechnology expands, buckminsterfullerene (C_{60}) is becoming more available and more affordable. For example, Frontier Carbon is producing fullerenes and carbon nanotubes in factories with capacities as high as 1,500 tons/year [1]. Thus, the potential for incidental and accidental releases of C_{60} to the environment is increasing. Because C_{60} is extremely insoluble in water (estimated solubility 1.3×10^{-11} g/L) [2], it is very likely that soils and sediments will eventually serve as a sink for released C_{60} nanoparticles. Thus, advancing our understanding of the potential hazards posed by C_{60} to the terrestrial biota is a priority for risk assessment.

Earthworms perform many essential and beneficial functions in terrestrial ecosystems, including decomposition, nutrient mineralization, and soil structure improvement [3], and these ecosystem services can be inhibited by the presence of toxic compounds. Earthworms are commonly used in toxicological studies because of their close contact with soils, thin and permeable cuticles, and large consumption of near-surface soil [4–11]. Earthworms are also essential for terrestrial food webs, so their response to engineered nanoparticles has important implications on potential impacts to terrestrial ecosystems.

Very few studies have considered the potential impact of C_{60} to terrestrial organisms, although some toxicological studies have been conducted with nC_{60} (stable water suspensions of aggregated C_{60}), which is generally considered as a different type of nanoparticle because of the different physicochemical and toxicological properties, such as size, color, hydrophobicity, and reactivity [12]. Tong et al. [13] showed that soil mitigates the bactericidal activity of nC_{60} that is commonly observed in low-salt mineral media [12,14–16]. Association of nC_{60} with soil decreases its bioavailability and thus diminishes its antibacterial activity [17]. Nevertheless, C_{60} stabilized with a polyvinylpyrrolidone coating was reported to kill mouse embryos at 137 mg/kg; the toxicity mechanism was attributed to hindering the function of the yolk sac and embryonic morphogenesis [18]. Other research has also indicated that nC_{60} delayed zebrafish embryo and larva development and exerted teratogenic effects [19]. Recent studies have shown that nC_{60} can be toxic to earthworms when present at 154 mg/kg soil, significantly reducing cocoon production by *Lumbricus rubellus* and increasing juvenile mortality rate [20]. Cocoon production by the earthworm *Eisenia veneta* was also observed to decrease in the presence of pristine C_{60} administered through food at 1,000 mg/kg [21].

Because earthworms can take up contaminants through skin contact as well as by soil ingestion, exposure scenarios in addition to food ingestion have to be considered to gain a more comprehensive understanding of the effects of C_{60} on earthworms. In the present study, we conducted acute soil avoidance tests and viability assays (reproduction and weight loss) to assess the potential toxicity of pristine C_{60} to the model earthworm *Esienia fetida*.

MATERIALS AND METHODS

Chemicals

Buckminsterfullerene (99.5% + pure) was purchased from SES Research. According to the manufacturer's data sheet, C_{60} has a ball outer diameter of 11 nm and a surface area of 1,520 nm^2, and its purity was 99.5% (the impurity is mainly C_{70}, with no heavy metals). Synthesis of C_{60} involved extraction of raw fullerene soot using various organic solvents to leave behind undissolvable material. The fullerenes were then isolated by different chromatographic methods, and purity was verified by high-performance liquid chromatography. Both $CaCO_3$ and KCl were purchased from Fisher Scientific.

Organisms

Eisenia fetida was purchased from The Worm Farm and maintained according to standard methods as described previously [22]. Sexually mature (i.e., clitellated) earthworms of 0.3 to 0.6 g were selected for all the experiments.

Artificial soil preparation and amendment

Artificial soil was prepared as described by Environment Canada [23]. It consists of 10% *Sphagnum* peat moss previously

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sieved through 2-mm mesh, 20% kaolin clay, and 70% quartz sand. The pH of dry soil was adjusted with $CaCO_3$ to the optimal range (6.5–7.5) and measured by mixing 10 g of soil with 50 ml of 0.01 M $CaCl_2$ solution. The mixture was allowed to settle for 1 h, and the pH was determined with a pH probe. C_{60} was amended into soil directly in the form of powder. Previous research shows that C_{60} powder can aggregate in soil and is unlikely to be transformed during the experimental period [22]. The soil mixtures were tumbled and mixed overnight. This approach achieves homogeneous mixing, as verified in previous studies with ^{14}C-labeled C_{60} [22]. Two concentrations of C_{60} were used in the avoidance test (2,000 and 10,000 mg/kg). Growth and reproduction tests, which require a smaller volume of soil and thus smaller amounts of C_{60}, were conducted with three C_{60} concentrations (5,000, 10,000, and 50,000 mg/kg). Note that avoidance is widely accepted as the most sensitive response of earthworms to chemicals because it generally occurs at concentrations lower than other toxicity thresholds such as weight loss, reproduction inhibition, or death [4,24]. To confirm the sensitivity of the avoidance test, phenanthrene (100 mg/kg) was added to separate treatments, as described previously by Li et al. [22,25], as a positive control.

Growth and reproduction tests

Growth and reproduction tests were conducted according to Environment Canada protocols [23]. After voiding gut contents on filter paper hydrated with deionized water for 24 h, earthworms were transferred to 500-ml glass jars filled with soil (10 worms/200 g soil) containing different concentrations of C_{60} (0, 5,000, 10,000, and 50,000 mg/kg) for 28 d. Soils in all treatments were hydrated with Milli-Q water to 85% of water-holding capacity (0.47 ml water/g soil). No earthworm food was provided during the exposure period to avoid confounding effects on C_{60} bioavailability caused by its potential association with the food, although the lack of food might have affected the sensitivity of earthworm growth and reproduction endpoints. Worm weight was measured and recorded at day 0 and day 28, after allowing the worms to void gut contents for 24 h. Cocoon numbers in each treatment were counted and recorded. All treatments were prepared in triplicate.

Avoidance test

Avoidance tests were conducted following a modified method developed by Environment Canada [23]. This test was conducted in stainless-steel avoidance wheels with six pie-shaped compartments connected to the center test arena (Fig. 1). Negative control soil (unamended artificial soil) was placed into alternating compartments (three compartments/unit, 250 g soil/compartment), and test soil was transferred to the remaining compartments. C_{60}-amended and control soils were separated by removable aluminum partitions. Soil in each compartment was hydrated with Milli-Q water to 85% of water-holding capacity. Partitions were removed afterward. Ten worms were selected for each avoidance wheel. Worms were introduced to the center of the avoidance wheel individually. After 10 worms had been added to an avoidance wheel, a lid was placed to prevent escape. At least five replicates were conducted for each treatment. The wheels were placed in a dark area at 22 °C for 48 h. At the end of 48 h, the lid was removed and the partitions were inserted back into the chambers to prevent further worm movement between the compartments. The number of worms in each compartment was recorded and the percentage of worms (10 in total) in C_{60}-amended and control soils was calculated.

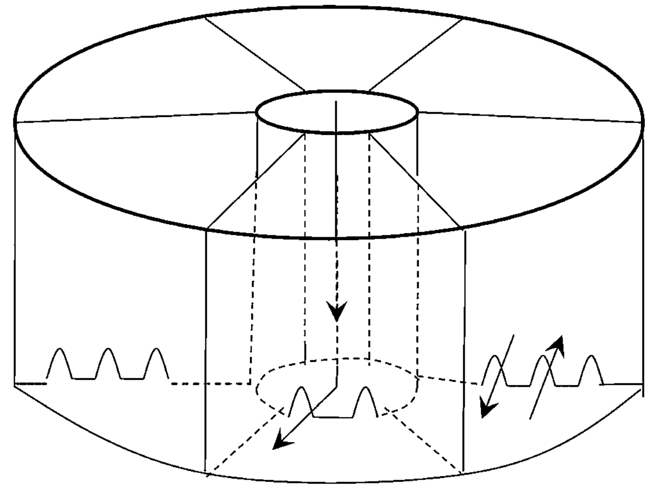

Fig. 1. Avoidance test wheel. Arrows illustrate how earthworms can move freely between compartments.

Statistical analysis

Whether differences in the percentage of worms in C_{60}-amended soils, worm weight loss, and cocoon numbers at specific C_{60} concentration were statistically discernible from control soils was determined by using analysis of variance coupled with Dunnett's post hoc test at the 95% confidence level [26].

RESULTS AND DISCUSSION

Similarly to a previous study with double-walled nanotubes administered through food at up to 495 mg/kg [21], which showed no earthworm mortality, no acute toxicity or earthworm mortality occurred with any treatment despite the relatively high C_{60} concentrations used in the present study. Earthworms did not significantly avoid ($p > 0.05$) soil amended with C_{60} powder at concentrations up to 10,000 mg/kg during the 48-h exposure period (Fig. 2). In contrast, significant avoidance of the positive control (phenanthrene at 100 mg/kg) occurred, demonstrating the sensitivity of this assay (Fig. 2). Higher C_{60} concentrations were not considered because of the large amount of C_{60} required for this avoidance test (and

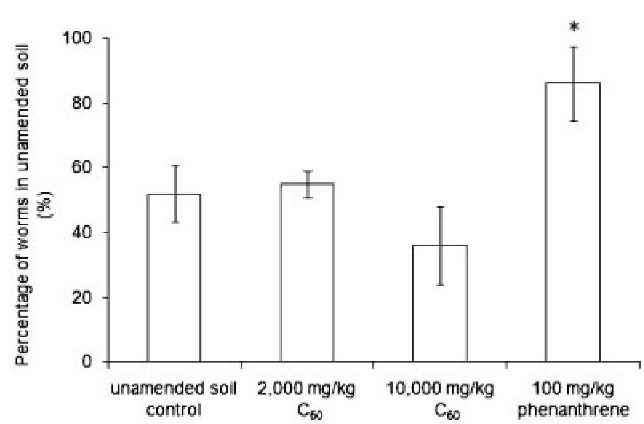

Fig. 2. Percentage of worms recovered in unamended soil compartments after the 48-h avoidance test. Worms did not significantly avoid soil amended with C_{60} at up to 10,000 mg/kg ($p > 0.05$) compared with control soil. Phenanthrene, the positive control, showed a significant avoidance effect at 100 mg/kg. The asterisk indicates a significant difference from controls at the 95% confidence level. Error bars represent ± standard error ($n = 5$).

the associated high cost) and the low probability that incidental releases of engineered nanoparticles would result in soil concentrations exceeding 1% [27].

Because food was not provided to earthworms during the standard 28-d growth and reproduction test, worms in control (unamended) soil lost $33 \pm 0.6\%$ of their total weight after a 28-d incubation (Fig. 3). Worms in soil amended with C_{60} (up to 50,000 mg/kg) experienced a similar, statistically indiscernible weight loss ($29 \pm 5.6\%$ for 5,000 mg/kg, $32 \pm 1.3\%$ for 10,000 mg/kg, and $22 \pm 5.2\%$ for 50,000 mg/kg, respectively).

At 50,000 mg/kg, C_{60} significantly decreased the earthworms' cocoon production ($p < 0.05$; Fig. 4). However, at lower concentrations (5,000 and 10,000 mg/kg), this effect was not statistically significant. Decreased reproduction was previously reported for earthworms exposed to food amended with a lower C_{60} concentration (1,000 mg/kg) and double-walled nanotubes (37 mg/kg) [21]. This likely reflects higher tolerance to C_{60} exposure in soil than through direct food administration. Although the mechanism by which C_{60} decreased earthworm reproduction is unclear, it is not likely to involve a clogged gut track, because no significant difference in weight loss was observed in earthworms exposed to C_{60} compared with unexposed controls (Fig. 3), and limited bioaccumulation of C_{60} occurred after 24 h of depuration, indicating unhindered ability to excrete C_{60} rapidly [22].

The high (1,000s of mg/kg) C_{60} concentrations used in this study to elicit a response are unlikely to be found in soils impacted by incidental releases [27]. Such high C_{60} concentrations would likely be found only at accidental release or disposal sites. The lack of significant effects (weight and population size) on *E. fetida* exposed to 10,000 mg/kg suggests that earthworms are unlikely to experience acute mortality as a result of the lower C_{60} concentrations expected to occur in soil. Nevertheless, the relative persistence of C_{60} [28] together with our finding that high C_{60} concentrations were not avoided but significantly hindered earthworm reproduction could be a cause of concern in chronic exposure scenarios. Earthworms have a relatively low reproduction rate (0.4 cocoon/day) [29], and a small decrease in this rate could significantly affect their population, potentially impacting the terrestrial food web. Therefore, longer exposure studies than the standard 28-d test are recommended to assess the potential chronic, sublethal effects of C_{60}.

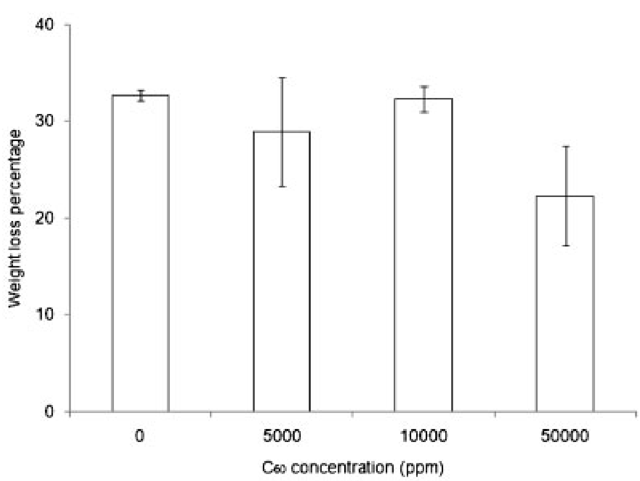

Fig. 3. Worm weight loss after a 28-d incubation in control soil or soils amended with C_{60}. The presence of C_{60} did not exert a significant effect on worm weight loss ($p > 0.05$). Error bars represent ± standard error ($n = 3$).

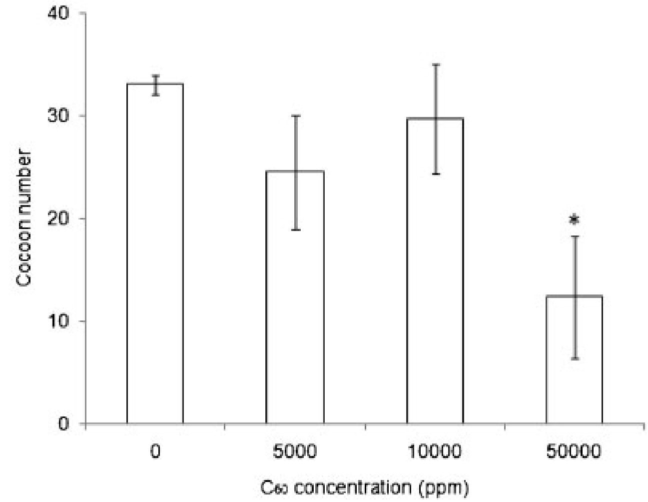

Fig. 4. Worm cocoon production after 28-d incubation in unamended soil or soils amended with C_{60}. At 50,000 mg/kg, C_{60} significantly decreased the cocoon production of earthworms. The asterisk indicates significant difference from controls at the 95% confidence level. Error bars represent ± standard error ($n = 3$).

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