Adverse effects of fullerenes ($nC_{60}$) spiked to sediments on *Lumbriculus variegatus* (Oligochaeta)

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**A B S T R A C T**

Effects of fullerene-spiked sediment on a benthic organism, *Lumbriculus variegatus* (Oligochaeta), were investigated. Survival, growth, reproduction, and feeding rates were measured to assess possible adverse effects of fullerene agglomerates produced by water stirring and then spiked to a natural sediment. *L. variegatus* were exposed to 10 and 50 mg fullerenes/kg sediment dry mass for 28 d. These concentrations did not impact worm survival or reproduction compared to the control. Feeding activities were slightly decreased for both concentrations indicating fullerenes' disruptive effect on feeding. Depuration efficiency decreased in the high concentration only. Electron and light microscopy and extraction of the worm fecal pellets revealed fullerene agglomerates in the gut tract but not absorption into gut epithelial cells. Micrographs also indicated that 16% of the epidermal cuticle fibers of the worms were not present in the 50 mg/kg exposures, which may make worms susceptible to other contaminants.

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1. Introduction

Carbon nanoparticles (NPs), such as fullerenes ($C_{60}$), are one of the most promising nanosized materials due to their versatile properties in electronics, optics, medicine, and cosmetics (Bakry et al., 2007; Degiorgi, 1998). Some of these uses, such as cosmetic products, suggest that they will be directly released to waste waters (Benn et al., 2011). Increasing manufacturing and use of fullerene-containing products may also cause fullerenes to enter the environment either from production of these products or their use by consumers. Similarly to other chemicals, there is no reason to assume that manufacturing and use of NPs can or will be a perfectly closed system without environmental release, and thus the potential ecological risks of fullerenes need to be understood.

Although fullerenes are highly insoluble in water (log $K_{ow} = 6.67$) (Jafvert and Kulkarni, 2008), they are modified during mixing in water to form stable agglomerates of many individual molecules, known as $nC_{60}$ (Scharff et al., 2004); $C_{60}$ is the most common type of fullerenes and thus the word “fullerene” is used hereafter to indicate $C_{60}$. This ability to be dispersed in water increases fullerene mobility in aquatic environments and causes them to be available to aquatic organisms (Deguchi et al., 2001). However, environmental parameters, such as water hardness, ionic strength, pH, and dissolved organic matter, strongly impact the agglomeration process (Ma and Bouchard, 2009; Isaacson et al., 2009; Brant et al., 2005). High concentrations of cations, especially divalent cations, lead to rapid agglomeration of $nC_{60}$ and settling out of solution (Chen and Elimelech, 2006), which will then make them available to benthic organisms.

Studies investigating fullerene toxicity to organisms in the water column have shown mixed results with some studies indicating only minor effects (Fraser et al., 2011; Tervonen et al., 2010), while other studies have shown long-term toxic effects such as reduced offspring production at a concentration of 2.5 mg/L (Tao et al., 2009). However, estimation of toxicity of fullerenes from the current literature is complicated, because many early studies showed toxic results that were later attributable to THF metabolites inadvertently formed in the THF dispersion method (Henry et al., 2011).

In contrast to the numerous studies on the toxic effects of fullerenes to organisms in the water column, no studies have been conducted yet on the effects of fullerenes to benthic organisms. These organisms have important ecological roles in aquatic ecosystems, two of which are as a food source for fish and as decomposers of sediment material. Additionally, Oligochaetes have been used as bioindicators of pollution (Lauritsen et al., 1985). For example, *Lumbricus variegatus* has been used in toxicity tests for decades (Bailey and Liu, 1980). Survival, growth, and reproduction are the most frequently used endpoints (Leppänen and Kukkonen, 1998a), and bioavailability tests have also been conducted (Leppänen and Kukkonen, 1998a,b,c). *L. variegatus* has been identified by the U.S. Environmental Protection Agency, OECD, and ASTM as a recommended freshwater organism.
for assessing bioaccumulation in sediments (U.S. EPA, 2000; OECD, 2007). One recent study on another oligochaete, terrestrial earthworm *Lumbricus rubellus*, showed that fullerene-spiked soils caused a serious population effect by altering mortality and the proportions of juveniles (van der Ploeg et al., 2011). Our hypothesis was that fullerene may similarly impact benthic species. *L. variegatus was chosen as a test benthic species due to its widespread usage in ecotoxicology testing as described above and its selective feeding on fine particulate matter (<63 μm) (Gaskel et al., 2007), the sediment fraction expected to contain fullerenes that have settled out of the water column.

The objective of this study was to assess the extent to which fullerene-spiked sediment may cause adverse effects on *L. variegatus*. To our knowledge, this is the first study on *L. variegatus* or any sediment-dwelling organism exposed to fullerenes. We spiked sediments with 10 and 50 mg fullerene/kg sediment dry mass and assessed toxicity using the following endpoints: mass change, reproduction, number of organisms, feeding rates, and morphological changes using transmission electron microscopy (TEM). Absorption of fullerenes into microvilli was also investigated using TEM.

2. Materials and methods

2.1. Chemicals

Fullerenes (C₆₀, 98%) were purchased from Sigma–Aldrich (USA). Artificial freshwater used to make the fullerene suspensions was prepared by adding analytical grade salts (Baker, Netherlands: CaCl₂, MgSO₄, NaNO₃, KCl, NaHCO₃, MgCl₂, NaOH, CH₃COONa, and Na₂CO₃) to Millipore-water (at least 18.2 mΩ) at 24.7 mg/L, NaHCO₃ 130.0 mg/L, KCl 1.2 mg/L, hardness [Ca²⁺ + Mg²⁺] = 5 mmol/L) to Millipore-water (at least 18.2 mΩ) and adjusting the pH to 6.8 (Tervonen et al., 2010). This artificial freshwater corresponded to Finnish lakes with its pH and hardness. NaCl (WVR International, France) and toluene (Baker, Holland) used in the extraction of fullerenes were analytical grade. Glutaraldehyde and Na-cacodylate used in electron microscopy samples were purchased from Merck (Germany) and Sigma–Aldrich (USA). Epon epoxy embedding medium was purchased from Fluka (Switzerland), Formvar polyvinyl resin for coating electron microscopy grids was purchased from SPI Supplies (U.S.).

2.2. Test organisms

*L. variegatus* originated from a U.S. EPA laboratory in Duluth, MN, USA. The *L. variegatus* culture was maintained at the University of Eastern Finland in 15 L tanks filled with artificial freshwater (1 mmol/L Ca²⁺ + Mg²⁺ hardness, pH 7). The tanks were continuously aerated to maintain the optimal oxygen content. The daily light–dark period for *L. variegatus* was 16:8 h, and they were fed with commercial fish food (Tetramin, Tetra Werke, Melle, Germany) twice weekly. The worms used pieces of pressed paper towel as a substrate, and water was renewed once a week. The temperature in the maintaining room was 20 ± 2 °C.

2.3. Preparation and characterization of the nC₆₀ suspension

Stock suspensions of nC₆₀ were made by mixing 100 mg of crystalline fullerene in 500 mL of artificial freshwater at 1000 rpm by magnetic stirring for 4 weeks at 20 ± 2 °C. Fullerenes were protected from light using an amber bottle and by wrapping the bottle with aluminum foil. The concentration of fullerenes in the suspension was analyzed by extracting fullerenes to toluene and measuring absorbance at 335 nm by a spectrophotometer (Cary 50 bio, Mulgrave, Australia) (Tervonen et al., 2010). The fullerene concentration in the stock suspension was 200 mg/L.

The shape and size of fullerenes in the stock solution was characterized by transmission electron microscopy. Eight microliters of the nC₆₀ suspension was placed on a Formvar coated 150-mesh copper grids (Leica, Wetzlar, Germany). Samples were then extracted twice to 5 mL toluene by bath sonication (Sonorex Super RK 106, Bandelin, Berlin, Germany) for 4 min, and then concentrated to 1 mL using an N-EVAP 112 nitrogen evaporator (Organomation Associates Inc., USA). A fullerene–specific peak at 335 nm (UV–VIS spectrophotometer Cary 50 bio, Mulgrave, Australia) and a standard curve (Tervonen et al., 2010) was used for fullerene quantification. Recovery of the extraction from the bulk sediment was 100 ± 14% (n = 3).

2.4. Test sediments

Natural sediment was collected from an 18-m depth from Lake Höytäväri (62° 41′ 21″ N, 29° 40′ 34″ E) close to the city of Joensuu in eastern Finland. The sediment was sieved to a particle size less than 1 mm and stored at 5 °C prior to testing. This sediment was chosen because it contains a sufficient amount of organic carbon for *L. variegatus* (Ristola et al., 1996a,b, 1999; Leppänen and Kukkonen, 1999a,b,c). It was collected from a clean area with concentrations of polycyclic aromatic hydrocarbons and polychlorinated biphenyls that were near or below the detection limits (Ristola et al., 1996a,b, 1999; Leppänen and Kukkonen, 1999a,b,c). The total organic carbon percentage, percentage of particles less than 63 μm, dry mass percentage, and pH were 3.6 ± 0.1, 57.3 ± 6.0, 21.6, and 7.0, respectively; uncertainties represent standard deviations of triplicate measurements.

The well-characterized fullerene suspension was spiked to 1000 g wet Lake Höytäväri sediment at nominal concentrations of 10 and 50 mg/kg sediment dry mass, and mixed for 4 h by vigorous stirring by a rotating metal blade. To our knowledge, there are no measurements of fullerene concentrations in real environmental sediments. While concentrations used in this study are higher than predicted and modeled average concentrations (Boxall et al., 2007; Gottschalk et al., 2009), it is common practice in microcosm studies to test higher concentrations than the expected average environmental concentration to investigate the possible toxic effects in hot spots with substantially elevated concentrations. A lack of an effect at higher concentrations suggests a lack of an effect at a lower concentration (Petersen et al., 2008). Lastly, the concentration range tested in this study is less than that recently tested by van der Ploeg et al. (2011) in soils (maximum concentration of 154 mg/kg) to investigate toxic effects to earthworms. Control sediment without fullerenes was similarly prepared. After spiking, sediments were stored for 4 d in the dark at 5 °C prior to starting the experiment.

Distribution of fullerenes in the sediment size fractions <63 μm and >63 μm was assessed by wet-sieving the spiked sediment and measuring fullerene contents in these fractions (three replicates for both fractions). For fullerene quantification, sediments were dried overnight in an oven at 105 °C, and then were pulverized in a mortar. The sediments were then transferred to 50 mL glass vials, homogenized twice in 5 mL 25% NaCl with a probe tip sonicator (Vibra Cell, Sonics & Materials Inc., Danbury, USA) for 4 min, and then extracted twice to 5 mL toluene by bath sonication (Sonorex Super RK 106, Bandelin, Berlin, Germany) for 15 min. Distinct fullerene phases were formed by centrifugation at 730 g for 1.5 min, the toluene phases from the two extractions were combined in separate vials, and then concentrated to 1 mL using an N-EVAP 112 nitrogen evaporator (Organomation Associates Inc., USA). A fullerene–specific peak at 335 nm (UV–VIS spectrophotometer Cary 50 bio, Mulgrave, Australia) and a standard curve (Tervonen et al., 2010) was used for fullerene quantification. Recovery of the extraction from the bulk sediment was 100 ± 14% (n = 3).

2.5. *L. variegatus exposure to test sediments

Test sediments (60 g w/w per container) were mixed, weighed, and added to 300 mL glass jars. Fifteen replicates were prepared at nominal dry mass concentrations of 10 and 50 mg/kg, and 15 control jars were also prepared. Then, 100 mL of artificial freshwater was gently added to the sediments to minimize re-suspension. Sediments were allowed to settle for one day, and then a few millimeter layer of combusted quartz sand (particle size 1–5 mm) was deposited onto the sediment surface to facilitate collection of fecal pellets (Leppänen and Kukkonen, 1998b). To ensure sufficient oxygen content in the overlying water, the samples were aerated via glass tubing inserted into the overlying water in each jar, and the jars were left dark at 5 °C prior to starting the experiment. Fifteen randomly chosen worms were added to each jar. The jars were covered with parafilm to reduce water evaporation. The temperature and daily light–dark period during experiments corresponded to the conditions described for the worm cultures. Behaviors of the worms were observed immediately after placing them into the jars and during the experiment. As is their typical behavior, the worms rapidly burrowed into and remained in the sediment throughout the experiment; their tunnels in the sediment were observed in all of the experimental conditions. The water volumes were checked in each container every two days during the experiment, and Millipore-water was added to replace evaporated water.

The feeding rate was determined using weighing fecal pellets (Leppänen and Kukkonen, 1999a,b,c). Changes in the feeding rate reflect the health of the population and the suitability of the sediment for them. This parameter was assessed by calculating the egestion activity as mg dry feces at each time point. For this purpose, the feces were collected with a pipette 4, 7, 14, 21, and 28 d after the beginning of the experiment. A sample of all the containers were sampled within a few minutes, it was not necessary to correct for the time difference among the containers during sampling. Water removed from the test jars caused by pipetting was replaced by adding a similar volume of artificial freshwater. Collected feces were vacuum-filtered through pre-weighted glass microfiber filters (pore size 1–2 μm, diameter 2.5 cm, Whatman, Maidstone, England). The filters on the feces were dried in an oven at 105 °C overnight, and then weighed with a microbalance (Sartorius 4503 Micro, Göttingen, Germany) and extracted to toluene by the method described for dried sediments above. For fullerene quantification, spectra for the tolune layers were recorded by UV–VIS spectrophotometer (Cary 50 bio, Mulgrave, Australia) from 700 to 280 nm. The high recovery of this method was previously checked with fullerenes is shown in Fig. S3. Pellets of the control worms did not yield peaks at the wavelengths specific for fullerenes. While the techniques described above were able to provide quantitative measurements of fullerenes in sediments and previous methods have been described for quantifying fullerenes in the crustacean Daphnia magna (Leppänen et al., 2010), these methods were not directly applicable.
applicable for detecting fullerene concentrations in _L. variegatus_ likely as a result of interference from other biomolecules such as lipids in the organisms (data not shown). The development of analytical methods to detect fullerenes in ecological receptors is a broadly recognized yet highly challenging direction for additional research (Isaachsen et al., 2009; Petersen and Henry, in press; Petersen et al., 2011), but not one pursued in this manuscript.

Effects on survival and reproduction were investigated by counting the worms after the 28 d exposure. Acute toxicity is a more sensitive endpoint than reproduction; therefore, a decrease in the number of worms during the experiment would indicate acute toxicity. If the number of worms increased during the exposure but the number of worms in the fullerene-spiked samples was less than that in the control samples, that would suggest decreased reproduction. However, worms decompose quickly in the sediment thus hindering unequivocal differentiation between decreased reproduction and mortality. Worm growth was estimated by comparing the changes in mass during the course of the experiment. Because _L. variegatus_ cannot be blotted dry and weighed without killing them, fourteen worms were weighed individually before the experiment, and the average was recorded as a “beginning mass”. After 28 d, the worms were collected from the sediment by wet-sieving. The worms were then transferred to 200 mL jars to clean artificial freshwater to dehydrate their gut content for 6 h (Mount et al., 1999). After depuration, a group of worms from ten randomly chosen jars per exposure concentration were carefully dried on blotting paper and weighed with a microbalance. Worms from the remaining five containers from each treatment were prepared for light and electron microscopy.

2.6. Light and electron microscopy studies for _L. variegatus_

Light microscopy was used to differentiate between fullerene particles and sediment particles in organisms’ guts. Ten horizontally and ten vertically cut 2 μm thick slides were prepared for light microscopy for each concentration. Slides were stained with toluidine blue to differentiate tissues and observed by 40×, 100×, and 400× magnifications (Leica CME, Buffalo, NY, USA). Electron microscopy analyses were conducted to investigate potential absorption of fullerenes and possible morphological changes in _L. variegatus_. After depuration in clean water for 6 h, 15 worms were collected randomly from five jars in each condition as mentioned above. The worms were placed on a petri dish in 1 mL of artificial freshwater. The heads of the worms were determined from the direction of movement, and then 1 mL of 4% glutaraldehyde fixative was allowed to mix with water to sacrifice the worms for fixing. The worms were immediately dissected to head and tail sections. These sections were then fixed with a 1:1 ratio of 4% glutaraldehyde and 0.2% Na-cadycolate buffer (pH 7.5) overnight in a refrigerator and then added to a Na-cadycolate buffer for at least 15 min. For post-fixation, worms were placed in a solution composed of a 1:1 ratio of 2% OsO4 and 0.2% 1 mol/L Na-cadycolate buffer (pH 7.5). Worms were then added to a fresh Na-cadycolate buffer for 15 min. Dehydration steps were made in an upward acetone series. Worms were placed consecutively in solutions of 30%, 60%, or 90% acetone for 10 min each, and then in 100% acetone for 10 min three times. The worms were then added for 30 min each to 1:1 solutions of acetone and Epon epoxy embedding medium. Infarilation to 100% Epon was made overnight, and the samples were then cast. After casting 80 nm thick slices were cut horizontally and vertically and placed on Formvar coated 150-mesh copper grids. Imaging was done operating at 50 kV incident beam energy with magnification ranging from 7000 to 50,000×. At least five grids per test concentration were observed for epidermis damages, and additionally five samples were used to investigate absorption in the gut. For assessing potential decreased reproduction, worm lengths were measured using a NanoMeasure Tool (Tokyo, Japan) operating at a 200 kV beam energy and with magnifications up to 90,000×.

2.7. Calculations and statistics

_SigmaPlot_ for Windows 11.0 (Systat Software, Inc., Germany) and SPSS 17.0 (IBM Corp., NY, USA) were used for statistical analyses. Shapiro–Wilkinson test was used to test for normality and Levene’s test was used to test for variance equality. One way ANOVA (with Dunnett _t_-test for post hoc comparison), ANCOVA, or ANOVA for repeated measurements (RM ANOVA) were used when data were normally distributed. RM ANOVA was required since the same replicate beamers were sampled for the fecal pellets over time and, thus, were not independent observations. Results were significant if _p_ < 0.05. Mauchly’s test of sphericity was conducted and Huynh–Feldt correction for the degrees of freedom was applied if the test was significant. When data were not normally distributed, Kruskal–Wallis one way ANOVA was applied.

3. Results

3.1. Survival, reproduction, and growth

_L. variegatus_ survived 28 d in all treatments, and there were no differences in burrowing behavior between control and spiked sediments. The average number of worms was 23.2 ± 2.5, 21.1 ± 4.0, and 21.6 ± 1.7 for the control worms and those exposed to 10 and 50 mg/kg dry mass, respectively; there was no significant difference in the number of worms between any groups (ANOVA). The average wet worm masses (mg) are presented in Table 1. There was no significant difference in the worm masses between the control and 10 mg/kg exposed worms, but control worms and those exposed to 50 mg/kg were significantly different (ANOVA, Dunnett _t_-test, _p_ = 0.008). Worms in both test concentrations and controls were lighter than the beginning mass (Fig. S4). It is common that worms lose weight during experiments due to poorer nutritional conditions compared to the maintaining conditions (Kukkonen and Landrum, 1994).

3.2. Electron and light microscopy for _L. variegatus_

Electron micrographs were investigated for worms that had been allowed to depurate for 6 h. Sediment particles were only found in the gut lumen in the tail sections of control and sediment and fullerene particles in the gut lumen of 10 mg/kg exposed worms. Both sediment and fullerene particles were also observed in the head section of worms exposed to 50 mg/kg (Fig. 1). Fullerenes and sediment particles could not be readily differentiated by TEM, thus light microscopy was used to reveal ingestion of fullerenes. Using light microscopy, fullerene particles appeared black, while sediment particles were light brown (Fig. 2). Black particles were observed only in exposed worms. Fullerenes agglomerates or any other particles were not found inside skin epithelial cells or in the gut microvilli by TEM. This result was confirmed using a separate TEM with higher resolution (see Fig. S5).

Possible morphological changes were investigated for the surfaces in contact with fullerenes: the epidermis and the gut. Depths of the cuticle layers covering the epithelial cells were measured (200 fibers in each treatment), and determined to be 138 ± 21, 135 ± 21, and 133 ± 22 nm for control, 10 mg/kg, and 50 mg/kg exposures, respectively. There were no differences in the cuticle fiber lengths (Kruskal–Wallis one way ANOVA). However, the cuticle fibers in the worms exposed to 50 mg/kg were not present in 16% of the surface (Fig. 3). Given that these measurements were made from numerous samples and that the cuticle fibers were always present in the control samples and worms exposed to 10 mg/kg, the absence of cuticle fibers is not believed to be an artifact from TEM sample preparation.

3.3. Feeding rate, depuration efficiency, and ingested fullerenes

Total amount of pellets produced in 28 days was higher in the control treatment (Table 1, ANOVA, Dunnett _t_-test: control vs. 10 mg/kg _p_ = 0.001 and vs. 50 mg/kg _p_ = 0.015). The mass of the worms was different between the control and the 50 mg/kg treatment (Table 1, ANOVA, Dunnett _t_-test _p_ = 0.008) at the end of the test. Because mass may have an influence on the pellet production (e.g. Leppänen and Kukkonen, 1998a,b,c), the dependence was studied by applying ANCOVA for the total pellet production (e.g. Leppänen and Kukkonen, 1998a,b,c), the dependence was studied by applying ANCOVA for the total pellet

<table>
<thead>
<tr>
<th>Amount of pellets (mg dw)</th>
<th>Average weight of worms</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1741 ± 119*</td>
</tr>
<tr>
<td>10 mg/kg dw</td>
<td>1345 ± 229</td>
</tr>
<tr>
<td>50 mg/kg dw</td>
<td>1462 ± 242</td>
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* Uncertainties are standard deviations.
production with mass as a covariate. Mass was not a significant variable ($p = 0.078$), and the treatment effect was also not significant ($p = 0.389$). However, a more powerful test was performed using data from all five sampling points (Fig. 4) that allowed the effect of time to influence through within-subjects factor in RM ANOVA. Time was significant ($p = 0.041$) and interacted with treatment factor (time $\times$ treatment, $p = 0.028$) and, again, mass was not significant ($p = 0.177$) but the treatment effect was significant ($p = 0.003$). Therefore, we did not use mass normalization in pellet production. Finally, RM ANOVA was performed without a covariate and the treatments differed significantly from the control (Dunnett $t$-test: control vs. 10 mg/kg $p = 0.001$, and vs. 50 mg/kg $p = 0.015$). Fecal pellet extractions revealed that they contained fullerene concentrations roughly twice that of the bulk sediment: $21 \pm 12$ mg/kg for nominal sediment concentrations 10 mg/kg and $110 \pm 16$ mg/kg for 50 mg/kg.

4. Discussion

Fullerenes did not affect the burrowing behavior of the worms or their survival and reproduction. This absence of a decrease in the number of worms accords with the lack of increased mortality previously observed for *L. variegatus* exposed to two other carbon nanoparticles, multi-walled carbon nanotubes (0.3 mg/kg) and single-walled carbon nanotubes (0.03 mg/kg) (Petersen et al., 2008a). Worms exposed to the highest fullerene concentration were smaller (on average 1 mg/worm ww) than those in the control condition and the 10 mg/kg exposure (see Fig. S4). Epidermal cuticle fibers were partly destroyed in worms exposed to 50 mg/kg dw. These cuticle fibers are reported to be a protective layer for heavy metals in another Oligochaeta (Maser and Rice, 1963), and they are assumed to have a similar function in *L. variegatus*. Destroyed fibers may increase the susceptibility of fullerene-exposed worms to other
contaminants or environmental stressors. However, this damage was observed only at 50 mg/kg, so this effect would not be expected for worms exposed to lower and more environmentally relevant fullerene concentrations. To our knowledge, there are no measurements of environmental concentrations of fullerenes from actual sediments, but fullerenes are modeled to reach sediments in concentrations 1–787 ng/kg/year (Gottschalk et al., 2009). For comparison of exposure concentrations and effects to a traditional pollutant, pyrene at a concentration of 0.18 mg/kg sediment dry mass caused a decreased egestion rate in the same sediment used in

Fig. 2. Particles observed by light microscopy. Fullerenes are black (three particles circled for an example), and sediment particles are brown and indicated by arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Epidermis and cuticle fibers of control worms (upper), and 50 mg/kg dw exposed worms (lower). Damaged fibers are marked by a bracket. Close-ups for normal (upper) and damaged epidermis (lower) at right. Scale bars are 2000 nm on the left and 500 nm on the right.

Fig. 4. Total pellet mass production during 28 days fullerene and control treatments. Sediments were spiked with 10 or 50 mg fullerenes/kg dry mass. Error bars represent standard deviations of ten replicates.
this study (Leppänen and Kukkonen, 1998c). Data was not available for comparisons to assess the sensitivity of many of the endpoints assessed in this study as a result of their novelty. The sensitivity of these endpoints and the suitability of various other compounds (i.e., organic chemicals, reference nanoparticles, or black carbon materials) for positive controls for sediment fullerenec ecotoxicity testing are important topics for additional research.

Both pellet extraction and electron and light microscopy revealed ingestion of fullerenes. In the control and 10 mg/kg exposed worms, particles were found only in the tail sections after 6 h of depuration indicating rapid excretion of ingested material, while both sediment and fullerenes were found also in the head sections in the worms exposed to 50 mg/kg. Particles remaining in the head sections of exposed worms indicated a decrease in depuration efficiency and the challenge of identifying absorption if only a few particles were absorbed in an entire organism. Thus, this result should not be taken as a conclusive indication that no fullerene absorption occurred, but rather that absorption was not observed using this technique.

Fullerene exposure appeared to decrease sediment egestion activity in both treatments based on statistical analysis. However, this effect is minor and can be found only when the full data set with several sampling points is tested. The results also indicate that the common practice of mass normalization in L. variegatus egestion activity studies should not be an automatic procedure. Researchers should first check the relationship between pellet production and worm mass. The lack of dependence can be related to typical architomic reproduction behavior of L. variegatus where splitting to two parts simply results in shorter and thus lighter worms. Therefore, normal three dimensional growth pattern from eggs and juveniles to adults does not take place and mainly the length of individuals changes. If the gut passage time per length unit remains the same, shorter worms may actually be more efficient pellet producers than heavier and longer ones (Leppänen and Kukkonen, 1998b).

Fullerene concentrations in fecal pellets were twice as high as in the bulk sediment. This could not be explained by fullerenes’ sorption to the finest fraction of the sediment; this fraction contained 57 ± 18% (n = 3) of the fullerenes in the sediment and was 51% of the total sediment. Alternatively, the higher fullerene concentration in the pellets may partly stem from the worms consuming and absorbing some fraction of the sediment for nutritional purposes while the fullerenes and other particles are excreted.

One potential implication of the worms concentrating fullerenes in their pellets is that bioturbation processes, whereby feeding by benthic organisms causes the re-suspension of contaminants (Könvalov et al., 2010), may also occur for settled fullerenes. Through this process previously settled fullerenes agglomerates may become more available to other benthic organisms and pelagic organisms in the water column.

Overall, exposing L. variegatus to environmentally high fullerene concentrations of 10 mg/kg and 50 mg/kg caused only minimal effects: decreased depuration efficiency and pellet production, smaller masses, and damaged cuticle fibers. These results accord with other studies where individual organisms have shown minimal acute effects from fullerenes (Fraser et al., 2011; Tervonen et al., 2010).

However, harmful effects of fullerenes have been observed at similar fullerene concentrations at the population level in earthworms (van der Ploeg et al., 2011). L. variegatus transferred fullerenes from the sediment to the sediment surface through feeding and egestion, thus potentially increasing the bioavailability of these fullerenes to epibenthic organisms that may be more sensitive to fullerene exposure, and further facilitate fullerene transfer in a food web.

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Appendix. Supplementary information

The supplementary information associated with this article can be found in the online version, at doi:10.1016/j.envpol.2011.07.014.

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