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TOXICITY OF FULLERENE (C_{60}) TO SEDIMENT-DWELLING INVERTEBRATE *CHIRONOMUS RIPARIUS* LARVAE

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Abstract—An environmentally realistic method to test fullerene (C_{60}) toxicity to the benthic organism *Chironomus riparius* was created by allowing suspended fullerenes to settle down, making a layer on top of the sediment. To test the hypothesis that higher food concentrations will reduce toxic responses, two food concentrations were tested (0.5 and 0.8% *Urtica* sp.) in sediment containing fullerene masses of 0.36 to 0.55 mg/cm^2 using a 10-d chronic test. In the 0.5% food level treatments, there were significant differences in all growth-related endpoints compared with controls. Fewer effects were observed for the higher food treatment. Fullerene agglomerates were observed by electron microscopy in the gut, but no absorption into the gut epithelial cells was detected. In the organisms exposed to fullerenes, microvilli were damaged and were significantly shorter. The potential toxicity of fullerene to *C. riparius* appears to be caused by morphological changes, inhibiting larval growth. Environ. Toxicol. Chem. 2012;31:2108–2116. © 2012 SETAC

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INTRODUCTION

The possible ecotoxicological risks of nanoparticles (NPs) have received increasing research attention in recent years as a result of the enormous economic potential of nanotechnology-related applications and the lack of information about such risks. The extent to which current standard ecotoxicological methods can be used to measure accurately the ecological risks of NPs is largely unknown because of the unique chemical properties of NPs, which substantially differ from those of hydrophobic organic chemicals and dissolved metals [1]. Test methods have recently been determined to be the most frequent topic in nanotoxicology for which scientists express uncertainty or incomplete knowledge [2].

Carbon-based NPs, such as fullerenes (C_{60}), are widely used in technological applications and commercial products [3,4], even though the risk assessment of such particles is still in its infancy, in part because of the lack of methods for quantifying their concentrations in environmental media [1,5,6]. Expected environmental releases of fullerenes into different ecosystems have been modeled [7]. However, the ecological risks that fullerenes may pose is unclear, partially because many early studies relied on solvent exchange methods to produce fullerene suspensions, such as with tetrahydrofuran (THF), a method that has been shown to produce toxic effects attributable to THF byproducts, not the fullerenes themselves [1,8–10]. The toxic effects of fullerenes themselves are not yet well understood, and standard methods have not yet been established for assessing their risks and interactions with other chemical or environmental compounds [6].

Fullerenes may exert toxic effects in aquatic ecosystems, where they may impact pelagic and benthic organisms. According to environmental modeling, sediments appear to be sinks for fullerenes in aquatic systems [7]. Although studies of carbon-based nanomaterials have investigated the potential toxic effects to aquatic organisms such as *Daphnia magna*, albeit often at high concentrations [6,11–13], the effects on benthic organisms are almost entirely unknown. Studies of carbon nanotubes and benthic invertebrates have been performed [14–18], but only a single study has been published on the effects of fullerenes on the benthic organism *Lumbriculus variegatus* [19].

Chironomus riparius are sediment-dwelling, deposit-feeding organisms, and their larvae are widely used in sediment toxicity experiments and as an environmental stress indicator [20–23]. The *C. riparius* larval life stage consists of four instars, with larvae living in the sediment in tubes that they have prepared using algae and sediment particles. Larvae use any suitable particles, such as algae, silt, and microdetritus as their food sources from the surface layer of the sediment [24,25]. Therefore, they are especially vulnerable to any chemicals and toxicants that have settled to the sediment surface and thus become available to larvae.

The aim of the present study was to investigate the extent to which fullerenes impact this benthic organism (*C. riparius*), using an abbreviated version of Organisation for Economic Co-operation and Development (OECD) standard method 218 [26] to test short-term effects after exposure for 10 d. First, a method for testing larval survival and growth was developed by designing an environmentally realistic exposure method that simulates a sensitive exposure route for *C. riparius*, given its feeding habits. All characteristics of the overlying water and sediment (e.g., pH, organic matter, ionic strength) must be taken into account when assessing the potential toxic effects of carbon NPs, and determining the influences of fullerenes in an artificial

All Supplemental Data may be found in the online version of this article.
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matrix represents an important first step. The present study used artificial freshwater and sediment. Suspensions of fullerenes (nC_{60}) were produced by stirring the water, the preparation method believed to be most environmentally relevant [27,28] and one that eliminated the possibility that byproducts from the suspension process could cause toxic responses. Second, two different food concentrations in the sediment were tested to investigate the impact of this parameter on toxic responses. This focus stems from documented research on *C. riparius* in which food quality is highlighted; growth and the capability to tolerate toxic compounds are improved when high quantities of food are available [29]. Thus, we hypothesized that higher food concentrations will decrease toxic effects.

MATERIALS AND METHODS

nC_{60} suspension and sediment characterization

Preparation of aqueous nC_{60} suspensions. Crystal fullerene power (purity 98%) was purchased from Sigma-Aldrich. Thermal gravimetric analysis (TA Instruments Q5000) and weighing of the platinum boat before and after heating with a microbalance were performed to analyze the samples. Samples of 1 to 2 mg were heated to temperatures above 750°C under a stream of air at 20 ml/min using a heating rate of 10°C per minute, and the mass remaining was consistently less than 0.1% ($n = 5$). Inductively coupled plasma optical emission spectrometry (ICP-OES; IRIS Intrepid ll XSP) analysis was additionally performed to trace possible metals (Supplemental Data, Table S1). Samples (0.5–0.7 g) were extracted with the MARS5-microwave digestion system, applying the U.S. Environmental Protection Agency (U.S. EPA)-3051 standard in which 6 ml HNO_3 and 1 ml H_2O_2 were used as an extraction acid (extraction volume 25 ml). Artificial freshwater was prepared by adding inorganic salts ($MgSO_4 \times 7\ H_2O$, $CaCl_2 \times H_2O$, KCl, and $NaHCO_3$, Ca + Mg hardness 0.5 mmol/L) [6] to Milli-Q water (>18.2 mΩ). The nC_{60} suspensions were made by adding approximately 400 mg crystalline fullerene (C_{60}) powder (purity 98%; Sigma-Aldrich) to 2 L artificial freshwater and stirring by a magnetic stir plate at 1,000 rad/min for two weeks in amber jars to prevent light interactions with the fullerenes. Concentrations of the suspension were determined before the experiments by using spectrophotometric measurements (UV/vis spectrophotometer; Cary 50BIO) [6]. Toluene was used to extract fullerenes from water, and, with toluene as a solvent, the maximum absorbance peak of C_{60} is 335 nm [30]. The absorbance value at that specific peak were used for quantitive C_{60} measurements and compared with the calibration curve (Supplemental Data, Fig. S1).

Characterization of fullerene agglomerates by transmission electron microscopy. The suspensions of nC_{60} were characterized by measuring their diameters using images from transmission electron microscopy (TEM; Zeiss 900, 50-kV incident beam energy). The largest dimension was used to indicate the particle size because the fullerenes were rarely spherical. Suspensions were not filtered, a decision intended to maintain the environmental similitude of exposure in which benthic organisms would likely be exposed to large fullerene agglomerates that settle to the sediment surface. Stock suspensions were diluted (1:30) with artificial freshwater to help decrease particle aggregation during the drying of the grids, and 8 μl of the dilution was added to Formvar (SPI Supplies) polyvinyl resin-coated 150-mesh copper grids (Leica). Before analyzing grids with a TEM (magnification range × 3,000–85,000), grids were air dried for 1 h and left in a desiccator overnight; two grids were analyzed from three different fullerene suspensions used in the experiments; overall, six grids were analyzed.

Preparation of the artificial sediment. Artificial sediment was prepared according to OECD method 315 [31] by mixing 22% kaolinite clay, 76% quartz sand (50% of the particles in the range of 50–200 μm), and 2% peat powder on a dry-mass basis. The pH of the sediment was adjusted to 6.25 with $CaCO_3$. Finely ground *Urtica* sp. leaves (particle size <0.5 mm) were added as a food source just before the experiment. Concentrations of *Urtica* were chosen according to artificial sediment preparation and storage recommendations [31], in which the food source used is recommended to be 0.5% (percentages of *Urtica* are always mass percentages with respect to the sediment dry mass). In addition, a higher food concentration (0.8% *Urtica* sp.) was chosen to investigate the possible difference in toxic responses of larvae under the favorable food circumstances. The dry mass to wet mass ratio of the sediment was determined to be 0.65 ± 0.004, and the total organic carbon content (TOC) was 2.18 ± 0.10 g/kg (Multi N/C 2100 Analytic Jena AG; uncertainties always represent standard deviations except for temperatures, in which case they indicate ranges).

Test organisms

Chironomus riparius were cultured in the laboratory of the University of Eastern Finland. Organisms had a light:dark period of 16:8 h in plastic aquaria at 20 ± 2°C. The aquaria contained a layer a few centimeters thick of natural lake sediment and 2 L artificial fresh water; a certain portion of water volume was replaced weekly. Approximately eight females and eight males from the culture basin were transferred to separated cages to mate in glass beakers filled with artificial fresh water for oviposition. All experiments started with first instars (≤3 d post hatch), and the midges were exposed individually.

Transmission electron microscopic observations of C. riparius

Transmission electron microscopy was used to assess the extent to which fullerenes were able to enter through the gut epithelia of *C. riparius* and to investigate internal structure changes in the organisms. In addition to analyzing the samples with the Zeiss 900 high-resolution TEM (JEOL-JEM 2100F) an incident beam energy of 200 kV was used to obtain higher resolution images and to confirm that the results obtained with the less powerful microscope were representative. The head, middle, and tail parts of larvae were used, and organisms were fixed with a 1:1 ratio of 4% glutaraldehyde:0.2% Na-cacodylate buffer (pH 7.5) overnight with refrigeration. Dehydration steps were made in an upward acetone concentration series. The larvae were consecutively placed in solutions of 30, 60, and 90% acetone for 10 min each, and then in 100% acetone for 10 min three times. The samples were then added for 30 min each to 1:1 and then 1:3 solutions of acetone and Epon. After infiltration to 100% Epon overnight, the samples were cast. Eighty–nanometer-thick slices were cut vertically in the area of the gut [32].

C. riparius *experiments*

Table 1 summarizes the test design parameters. The purpose of experiment 1 was twofold: first, to produce samples for the TEM analyses, and second, to determine the mean and variance for control and fullerene treatments. These statistical parameters were applied to calculate the required sample size in experiments 2 and 3 to make our test capable of detecting effects in subsequent statistical analysis. Experiments 2 and 3 tested the extent to which food concentration would impact

Table 1. Test design

	Experiment 1	Experiment 2	Experiment 3
No. of replicates (glass beakers)	40	30	30
Food source (*Urtica* sp.) mass percentage with respect to the sediment dry mass	0.8	0.5	0.8
Concentration of used fullerene suspension (mg/ml)	146.4/172.5[a]	143.7	160.7
Average surface density of settled fullerene in glass beakers (mg/cm^2)	0.55 ± 0.11[b]	0.36 ± 0.05	0.44 ± 0.04
Measured mean pH at the end of the test, control/exposed	$8.6 \pm 0.1/8.4 \pm 0.1$	$8.2 \pm 0.1/7.9 \pm 0.1$	$8.7 \pm 0.1/8.5 \pm 0.1$
Measured ammonia concentrations at the end of the test (mg NH_4+/L), control/exposed	5.1/6.2	0.6/1.1	2.9/4.1

[a] Two nC_{60} suspensions were needed, given the larger volume of solution and the larger number of replicates.
[b] Uncertainties represent standard deviations.

fullerene toxicity. The fullerene exposure method used in the present study resulted in fullerene agglomerates settling on the sediment surface layer at average surface densities of 0.36 to 0.55 mg/cm^2. The fullerene surface densities were determined by measuring the concentration in the aqueous phase before and after the settling period. The sediment was not directly tested. The concentrations tested in the present study are substantially higher than what would be expected in the environment, on average, to test for potential effects in hot spots with elevated concentrations. A lack of an effect at these higher concentrations also suggests a lack of an effect at lower and more environmentally relevant concentrations [33].

Experiment 1: Exposure method for C. riparius and TEM samples. In each 50-ml beaker (bottom surface area 9.60 cm^2), 10 g wet artificial sediment and 40 ml nC_{60} suspensions were added. A food source (0.8% *Urtica* powder) was mixed in the sediment prior to adding it to the beakers. Fullerenes were allowed to settle on the surface layer of the sediment for 2 d, thus imitating the expected environmental conditions for water bodies with fullerenes initially suspended in the aqueous phase. This process created a thin layer of settled fullerenes above the sediment, which is especially important for *C. riparius* because the larvae use particles from the sediment surface for nutrition. The fullerene suspension (35 ml) was then removed and replaced carefully with 40 ml artificial fresh water, and the beakers were undisturbed for 1 d, after which aeration was started. The water:sediment ratio was approximately 1:4. To assess the amount of fullerene that settled onto the sediment, the concentration of nC_{60} in the removed suspensions from 10 beakers was measured by extraction into toluene with spectrophotometric measurements, and the mass of settled fullerenes was determined by a mass balance. The density of fullerenes on the sediment surface was 0.55 ± 0.11 mg/cm^2 for this experiment, indicating that a relatively homogeneous distribution between the replicates was obtained. One larva in each beaker was carefully added with a Pasteur pipette under the water surface, while the aeration was stopped.

Test conditions such as pH, dissolved oxygen content, and temperature were recorded (measured by a MultiLine F/SET-3 universal meter) at the beginning and end of the experiment from the same 10 beakers in both exposure and control conditions. Ammonia (NH_4+), a compound that may cause toxic effects to organisms if present at high concentrations under alkaline conditions [34], was tested as a composite sample at the conclusions of the experiments, and the values were below those that would raise concerns about potentially harmful effects. Measurements of the overlying water were in accordance with the chironomid toxicity test guideline limits in all experiments: pH within the range of 6 to 9 and dissolved oxygen content >60% air saturation value (OECD, 2004) [26]. Water temperature did not differ by more than ± 1.0°C.

At the conclusion of the experiments, larvae were sieved (200-μm sieve), and surviving larvae were counted after they had been preserved in ethanol. Endpoints measured using a stereomicroscope (Nikon SMS 800) were body length (mm) and head capsules width and length (mm), and larvae wet mass and dry mass (measured by heating at 100°C until no further mass changes were observed) were measured by using a microbalance (Sartorius 4503 micro). Altogether, 11 individuals (five from control beakers and six from beakers in which they were exposed to fullerenes) for TEM analysis were collected, and samples from the head, middle, and tail parts were prepared separately. Overall, 132 sections were examined via TEM. Thick slices (2 μm), which were stained with toluidine blue, were made from the same sections used for light microscopic observations (observed at ×40, ×100, and ×400 magnifications; Leica CME) of larval internal structure. Images were taken with an Olympus DP-12 microscope digital camera system.

Experiments 2 and 3: Larval survival and growth in a 10-d chronic test. Chironomus riparius were exposed in sediments with fullerenes using two different food concentrations; experiment 2 used 0.5% *Urtica* in accordance with OECD method 218 [31], and experiment 3 used an increased concentration of 0.8%. Control containers with the same food concentrations but without fullerenes were also prepared. Thirty organisms were tested for both food concentrations and for controls. The volume of the nC_{60} suspensions in each beaker was lowered to 25 ml to decrease the mass of fullerenes used, and this solution was removed after 2 d of settling. The measured fullerenes surface densities in experiments 2 and 3 were 0.36 ± 0.05 and 0.44 ± 0.04 mg/cm^2, respectively. The same artificial sediment and freshwater were used as in experiment 1. Light and temperature conditions were the same as for the culture aquaria. Aeration was maintained during the experiments, and water lost by evaporation was replaced by Milli-Q water. Water-quality parameters were also measured on day 5. In experiment 3, one larva in the exposed treatment was found to reach the pupal stage, and its measurements were excluded from further analyses. Mortality was not observed in any experiments.

Data assessment

Data were analyzed with SigmaPlot for Windows 11.0 (Systat Software). GraphPad Prism 5 for Windows (GraphPad Software) was used for producing graphs. Responses in fullerene treatments were compared with controls by applying paired sample *t* tests if normality was confirmed by the Shapiro–Wilkinson test. When data were not normally distributed, the Mann–Whitney test was performed instead. Results were indicated as being statistically significant at $p < 0.05$.

RESULTS

Characterization of fullerene agglomerates by transmission electron microscopy

Images from TEM measured from the four suspensions showed a fullerene agglomerate size distribution ranging from 20 to 3,620 nm. The average particle size was 490 ± 475 nm ($n = 600$; Fig. 1A). Most agglomerates were between 100 and 200 nm, which included 20% of the agglomerates; In the suggested terminology of Taurozzi et al. (2011), the term "agglomerates" is used instead of "aggregates," because these particles would likely be broken apart to some extent by high-intensity sonication. The largest agglomerate sizes (>1,000 nm) were 10% of the distribution, a portion that most likely settled onto the sediment. Agglomerates were observed to be roughly spherical or cubical (Fig. 1B).

Light and TEM observations of C. riparius

Fullerene agglomerates could not be identified in the *C. riparius* internal structures by using light microscopy, but the gut wall, microvilli layer, peritrophic membrane, and ingested food in the gut could be observed (Fig. 2A,B). In exposed samples, the gut was almost completely full of food material, whereas, in the controls, the gut was not as fully packed. This created a larger area between the peritrophic membrane and microvilli layer, a space called the "ectoperitrophic space" (ETS) for the control organisms.

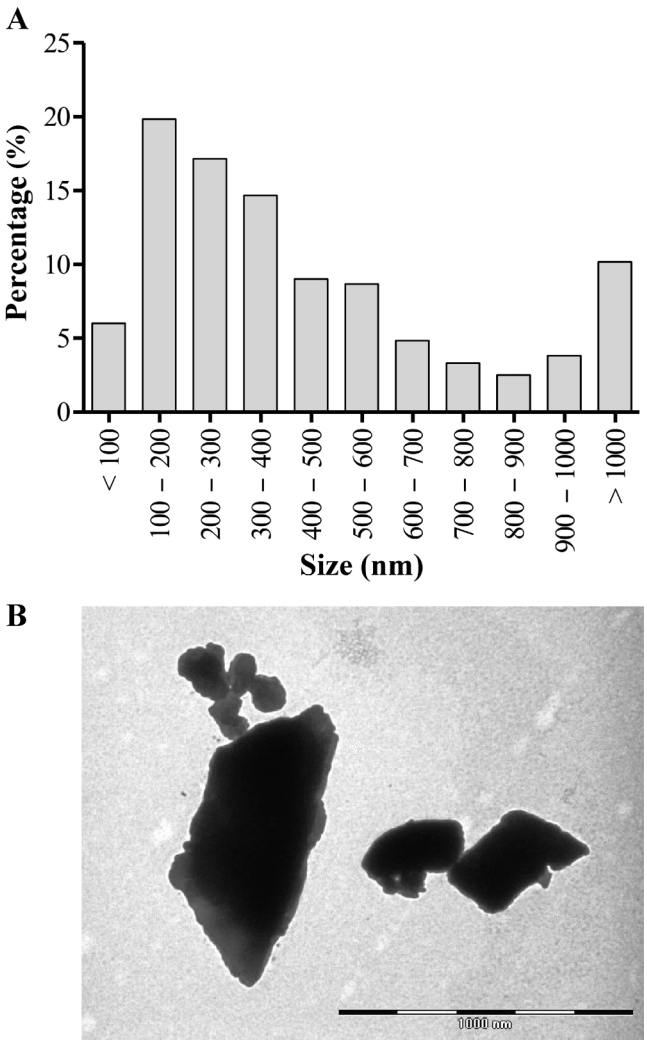

Fig. 1. Characterization of fullerene aggregates. (**A**) Histogram of suspended fullerene aggregates ($n = 600$). (**B**) Representative transmission electron micrograph image of nC_{60} agglomerates (×20,000).

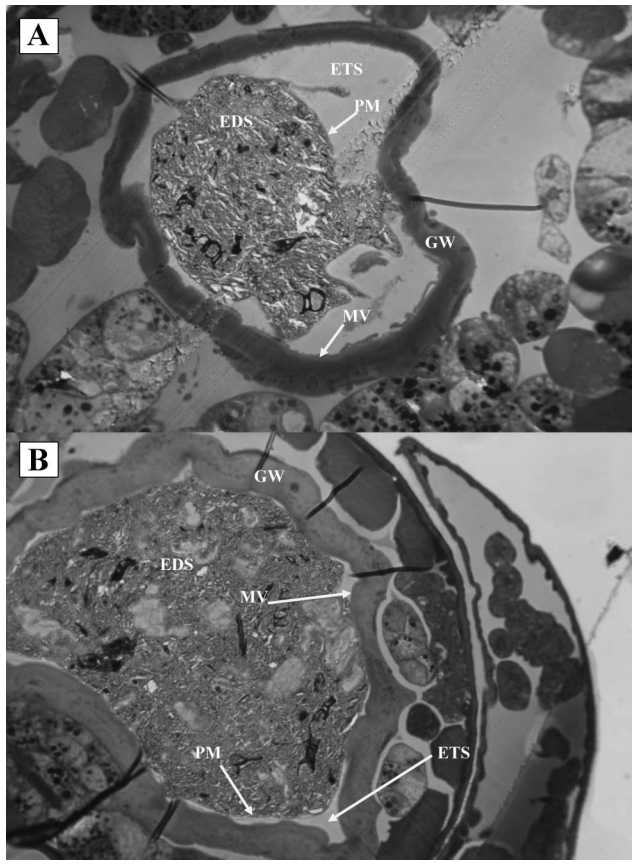

Fig. 2. Light micrographs of the midgut (×20). (**A**) Thick slice of the internal structure from a control organism and (**B**) from an organism exposed to fullerenes at an average surface density of 0.55 mg/cm^2 and a food source of 0.8% (*Urtica* sp.). PM = peritrophic membrane filled with food material; GW = gut wall; ETS = ectoperitrophic space; EDS = endoperitrophic space; MV = microvilli.

Transmission electron microscopic samples of the head, middle, and tail parts showed that the gut structure, peritrophic membrane, and microvilli were visible (Fig. 3A–F). Differences in microvilli structure were observed between the control and the exposed organisms. The microvilli were denser in the control samples, whereas exposed samples showed blank areas of microvilli (Fig. 3A–D), but this absence of microvilli was not observed in the tail parts (Fig. 3E,F). Transmission electron microscopy also revealed the presence of what appeared to be fullerene agglomerates in all larvae sections of exposed organisms (Fig. 3B,D,F). High-resolution TEM samples from the exposed head part sample showed that nC_{60} agglomerates were tightly packed on both sides of the peritrophic membrane and sometimes seemed to have broken through it (Fig. 4A,B). Absorption of fullerene particles into the microvilli was not detected in any sample with either electron microscope. Microvilli of the control organisms were shown to be significantly longer throughout the gut (Fig. 5) for the head, middle, and tail parts (Mann–Whitney test: $p < 0.0001$, $n = 200$). The most substantial difference was observed in the middle part, where the average microvilli lengths were $1{,}898 \pm 360$ nm and 467 ± 74 nm for the control and exposed organisms, respectively.

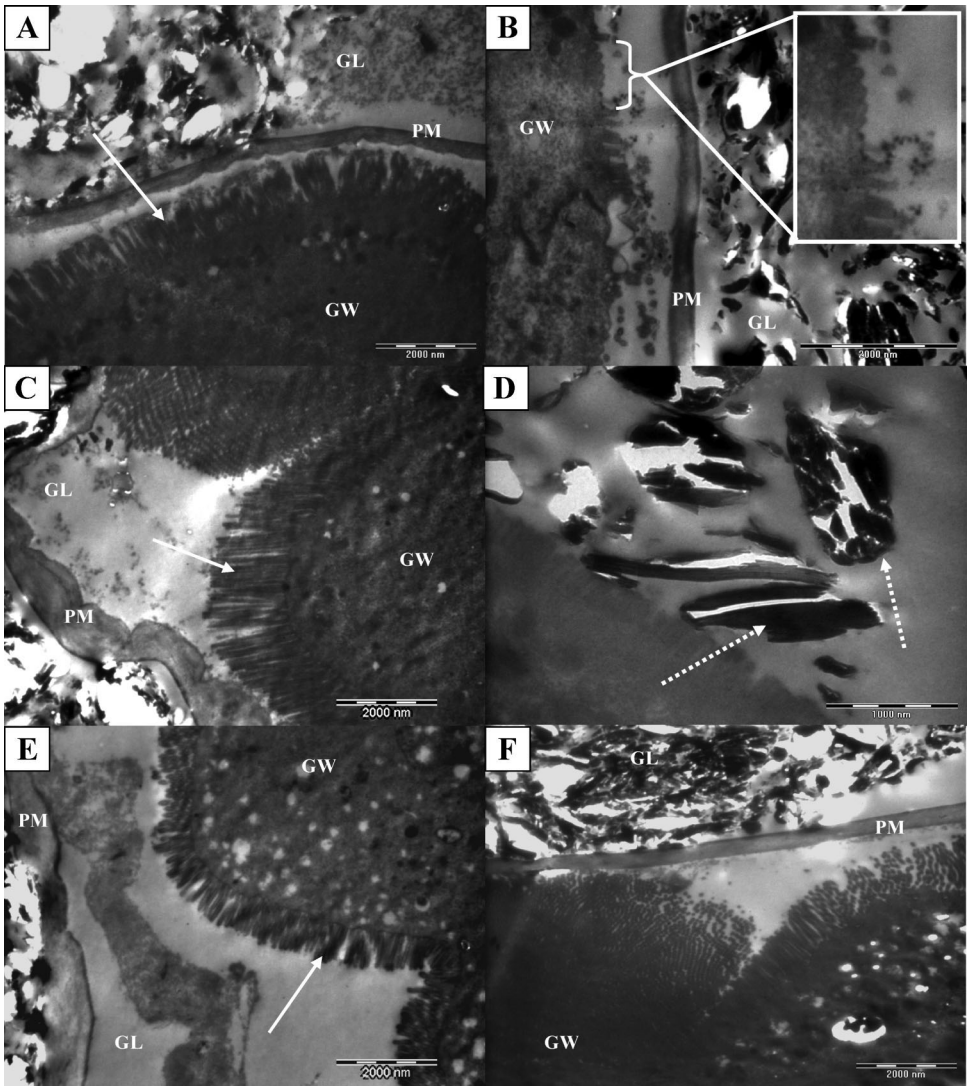

Fig. 3. Transmission electron micrographs of *Chironomus riparius*. (**A**) Image of the control head part gut sample of *C. riparius* (×4,400). (**B**) An exposed head part sample with microvilli missing (×7,000). (**C**) Long and dense microvilli structure in the controls middle part of the gut (×3,000). (**D**) Microvilli are visible shorter in the exposed middle parts samples (×12,000). (**E**) Tail parts microvilli structure in the control (×3,000) and (**F**) in the exposed samples (×4,400). GW = gut wall; GL= gut lumen; PM = peritrophic membrane. Solid arrows indicate microvilli; dashed arrows indicate nC_{60} agglomerates.

Larval growth in the 10-d chronic test

Significant differences between the control and the exposed organisms were found in all endpoint parameters in experiment 2 (see Fig. 6). Based on the head capsule length, four larvae exposed to fullerenes did not reach the fourth instar, whereas, in the control treatments, only one larva was in third instar (Fig. 6D,E), indicating delayed development for the exposed organisms. The mean head capsule length for the control larvae was 0.73 ± 0.09 mm and 0.66 ± 0.11 mm for the exposed organisms, a significant decrease ($p = 0.0016$, $n = 28$, Mann–Whitney test). Fullerene treatment also caused a statistically significant decrease in body length ($p = 0.0266$, $n = 29$, t test; Fig. 6C). In contrast, there was no statistically significant difference in body length or head capsule width measurements for the organisms with 0.8% *Urtica* in the sediment in experiment 3 (Fig. 6). In both experiments, fullerene exposure did not affect the organism dry mass, but there was a significant decrease in the wet mass measurements ($p = 0.003$, $n = 29$ [experiment 2] or $p = 0.006$, $n = 28$ [experiment 3], Mann–Whitney test; Fig. 6A,B). The highest ammonia concentration measured in the present study was in the exposure treatment in experiment 3 and was 6.2 mg/L, an amount much lower than 18 mg/L, which reportedly did not cause harmful effects on development or emergence of *C. riparius* [34].

DISCUSSION

In the present study, fullerene agglomerates caused morphological changes in the gut and microvilli as determined by light and transmission electron microscopy. Such effects on the internal structures of the organisms are the expected causes of the observed decreased larval, growth, and inhibited growth was also observed for *C. tentans* exposed to various metal oxide NPs above threshold concentrations [35]. In the present study, significant differences were measured for all endpoint parameters for a standard sediment food concentration of 0.5% (*Urtica*), whereas less pronounced effects were observed in higher food concentrations; because fullerene assimilation was not observed, fullerenes are not expected to have any nutritional value. This result is in line with our hypothesis that higher food concentrations would mitigate the effects of fullerenes as a

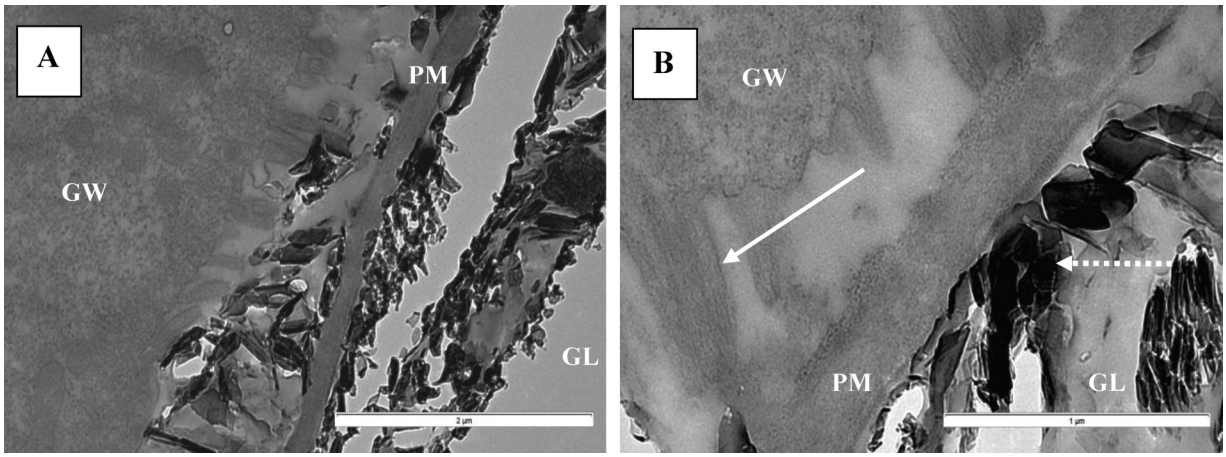

Fig. 4. High-resolution electron micrographs of *Chironomus riparius* in the exposed head part sample. (**A**) nC_{60} agglomerates are packed tightly against the PM. (**B**) Structure of microvilli and the PM shown in greater detail. Scale bars = 2 μm in A; 1 μm in B. GW= gut wall; GL= gut lumen; PM= peritrophic membrane. Solid arrows indicate microvilli; dashed arrows indicate nC_{60} agglomerates.

result of the higher nutrient availability and improved organism health. It was recently shown that feeding was necessary for a pelagic organism, *D. magna*, to eliminate multiwalled carbon nanotubes [33,36], so increased food concentrations may decrease the retention time of fullerenes in the organism's digestive system.

According to light microscopic images, the ingested material seemed to be more densely compacted in the guts of the organisms exposed to fullerenes. Transmission electron microscopy indicated that fullerene agglomerates were present in these compacted gut areas. The apparent toxicological consequence of the more densely packed materials in the guts is that the microvilli were shortened, and areas were observed without the microvilli layer, indicating damage to the gut epithelia. Because this was not observed in control organisms, sections without the microvilli layer are not believed to be an artifact of the TEM sample preparation method. Given that the microvilli in the middle section were the most sensitive to physiological changes from fullerene exposure according to TEM analysis, it may be sufficient to focus on this section in future studies. The dense packaging of fullerene agglomerates might also affect functioning of the peritrophic membrane, which helps to improve digestion and protects the gut epithelium [37] by causing pressure and thus rupturing the epithelial layer [38,39]. This effect is ecologically important, because organisms with damaged peritrophic membranes are more vulnerable to other chemicals and infections [37]. Evidence for peritrophic membrane damage and particles in contact with the epithelial layer were observed only in the organisms exposed to fullerenes. This was more prevalent in the middle gut sections, where the whole peritrophic membrane was occasionally removed and agglomerates were in contact with the microvilli layer. The peritrophic membrane structure in larval *C. tentans* consists of only one layer in the midgut but two or three layers in other parts of the organism [38]. If this is the case in *C. riparius* as well, it could explain why the middle gut sections showed substantially more damage than the other sections. Nonetheless, more research on the peritrophic membrane layer in *C. riparius* is needed to confirm this. Regardless, these results strongly suggest that the most likely mechanism for *C. riparius* damage is physical changes caused by peritrophic membrane and subsequently microvilli damage. This may adversely impact the organisms by hindering absorption of food and nutrients. Despite the damaged peritrophic membranes, uptake of fullerenes into the gut epithelial cells was not evident, which is similar to previous results for carbon NP uptake in crustaceans [6,13,40].

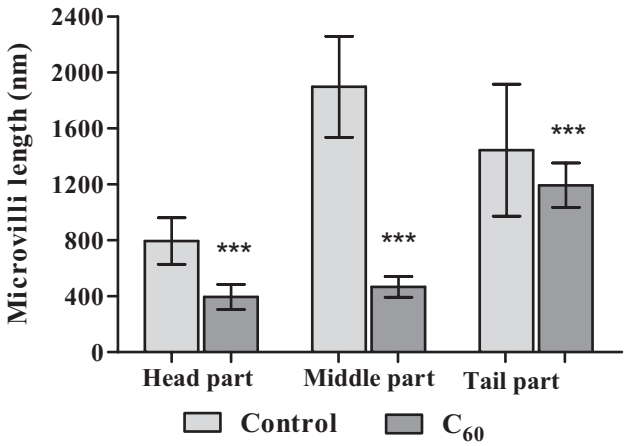

Fig. 5. Mean values for microvilli length ($n = 200$) in control and exposed samples from the head, middle, and tail parts of the larvae. Error bars are the standard deviations. Asterisks indicate significant differences from controls (Mann–Whitney test, $^{***}p < 0.0001$).

One of the primary findings of the present study is that food concentration strongly impacted the toxic effects observed and is likely an important parameter for nanoecotoxicology testing in sediments. There is a direct connection between the growth of Chironomidae and the quality and quantity of food [41], which thus impacts sediment toxicity tests. Higher concentrations of food in sediments cause the growth and reproduction of *C. riparius* to increase, and they populate sediments more densely even in the presence of toxicants relative to sediments with less food available [29]. Given that the body lengths, for example, were significantly longer ($p < 0.001$, $n = 29$, Mann–Whitney test) in the organisms exposed to C_{60} in experiment 3 compared with the control organisms in experiment 2, the previous finding mirrors the results observed here. The organisms exposed to fullerenes showed decreased toxic responses relative to control organisms at higher food levels, which suggests that organisms in ecosystems will display fewer effects from fullerene exposure in nutrient-rich environments. The addition of food to sediments could be a relatively straightforward approach to mitigate potential harmful effects in the event of NP contamination in sediments. Nevertheless, an important

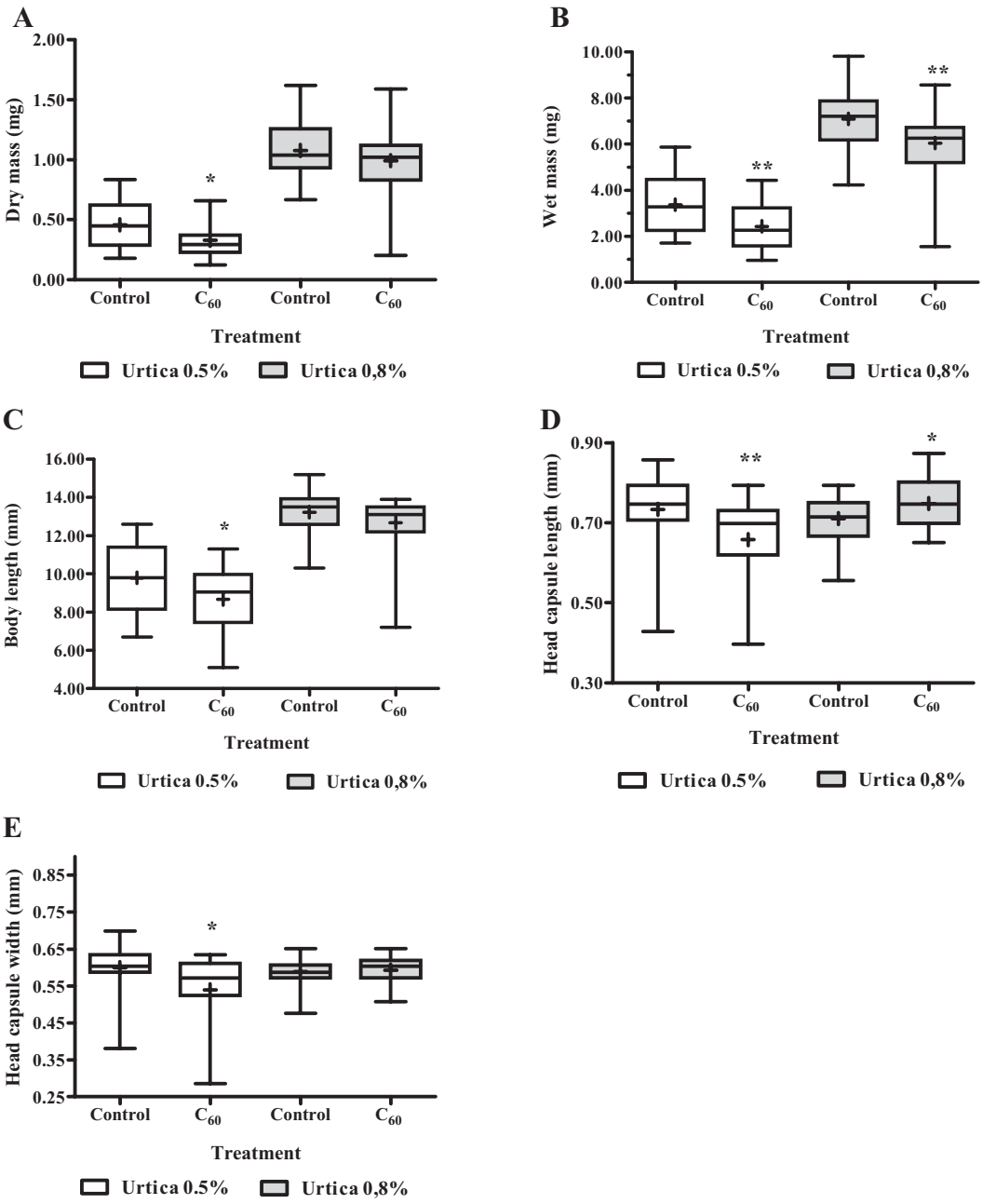

Fig. 6. Box plots of the results for larval survival and growth in a 10-d chronic test in experiments 2 and 3. (**A**) Dry mass (mg), (**B**) wet mass (mg), (**C**) body length (mm), (**D**) head capsule length (mm), (**E**) head capsule width (mm). For all parts in this figure, $n = 29$ and $n = 28$ for experiments 2 and 3, respectively. For symbols, (+) indicates mean values, whiskers indicate minimum and maximum values, and asterisks indicate significant differences from controls (*t* test or Mann–Whitney test, $^{*}p < 0.05$, $^{**}p < 0.001$).

topic for future research is to test the impact of additional organic material other than a food source to investigate how that might affect the toxic parameters measured here. With regard to the development of standard methods for nanoecotoxicology testing in sediments, particular care should be given to the recommended food concentration to be used with chironomids because this choice may influence the apparent toxicity or lack thereof for NP-spiked sediments. This research also suggests that testing sediment with a range of food concentrations and sources representative of those present in natural sediments or testing sediments from the natural environment is needed to understand fully the role of food in sediment nanoecotoxicology. Also, the mechanisms of how NPs affect benthic organisms are not yet well understood. One potential approach to investigate the mechanisms more fully in future studies is to include a hard-carbon control (e.g., activated carbon or charcoal) to investigate whether the toxicity caused by the fullerenes is a result of additional hard carbons being added to the sediment [1,36].

An additional contribution of the present study with regard to the development of standard methods for ecotoxicity testing is allowing the NPs to settle onto the sediment rather than directly spiking them to the full sediment as is typical for hydrophobic organic chemicals. This settling method was expected to represent a sensitive exposure for *C. riparius* larvae as a result of their use of particles on the sediment surface for feeding. In a separate study, fullene aggregates were spiked into the sediment, and the fullerene aggregates were shown to have

minimal toxic effects at concentrations of 10 and 50 mg/kg in oligochaete *L. variegatus* [19]. Given that processes such as bioturbation and scouring will resuspend sediment, it is important to test the toxic effects of fullerene aggregates on the surface for organisms especially sensitive to contamination on the sediment surface. Nevertheless, preparation of NP suspensions and how they should be spiked to soils, sediments, or food is an ongoing topic for investigation in NP testing [16,42]. Unlike the case for hydrophobic organic chemicals that are already present in sediments in the natural environment and for which extraction techniques are typically available, the concentrations of fullerenes in various environmental matrices are unknown, and quantification techniques are still being developed [5]; thus, concentrations of fullerenes in the organisms were not measured in the present study. An important topic for future research is to determine the impact of different fullerene spiking approaches on the risks that they pose in sediments and to conduct a dose–response test to examine the impact of a range of fullerene concentrations, including concentrations more environmentally realistic on these endpoints.

CONCLUSIONS

The present study included the design of a realistic exposure method for assessing toxicity of fullerene (C_{60}) to benthic invertebrate *C. riparius*, providing the first data on the ecotoxicological risks of fullerene agglomerates in sediments, showing that food concentrations are an important topic in test methods for fullerenes, and suggesting that physical damage in the digestive tract is the mechanism for inhibited growth. Nonetheless, many challenges remain, such as the development of a method for quantification of fullerenes in larger organisms and NP characterization of the fullerenes in sediments [1]. The present study provides an important first step toward the development of standard ecotoxicological methods for fullerenes and understanding the potential risks that these materials may carry after release into the natural environment.

SUPPLEMENTAL DATA

Table S1. Inductively coupled plasma optical emission spectrometric (ICP-OES, IRIS Intrepid ll XSP) analysis of fullerenes (C_{60}).

Fig. S1. Calibration curve of fullerenes (C_{60}) in toluene. (49 KB DOC)

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