``Changes in agglomeration of C_{60} and C_{70} Resulting from Accumulation and Depuration in Thamnocephalus platuyrus

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Exposure of the crustacean *Thamnocephalus platyurus* to stable aqueous suspensions of fullerenes C_{60} and C_{70} were studied. Aqueous fullerene suspensions were formed after stirring of C_{60} and C_{70} "as received" from a commercial vendor in deionized water (termed aqu/C_{60} and aqu/C_{70}) for approximately 100 days. The z-average diameters of aqu/C_{60} and aqu/C_{70} aggregates as measured by dynamic light scattering were 517 ± 21 nm and 656 ± 39 nm (mean ± 95% confidence limit), respectively. Exposure of *T. platryurus* to fullerene suspensions resulted in the formation of dark masses in the digestive track visible under a stereo microscope (40 x magnification). To confirm fullerene accumulation, the organisms were extracted and extracts analyzed by HPLC-UV and HPLC-MS. One hour exposures resulted in accumulations for aqu/C_{60} and aqu/C_{70} of 87 ng/organism and 345 ng/organism, respectively. Thin section TEM of aqu/C_{60}-exposed *T. platyurus* showed larger fullerene agglomerates than were observed prior to ingestion. As observed under light microscopy, these larger agglomerates appeared to clump together in the excretion pellets as compared to the indicator microspheres which dispersed after excretion. After depuration, the aqu/C_{60} settled to the bottom of the incubation wells and remained agglomerated for over 1 month. The agglomerates could be dispersed by sonication to form aggregates with an average Z-average of 273±16 nm.

Key words: Nanomaterials, Fullerenes, C_{60}, C_{70}, *Thamnocephalus platyurus*.

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Introduction

Nanotechnology is a rapidly growing industrial enterprise encompassing a diverse array of engineered nanomaterials, developed and applied in fields such as medicine, plastics, energy, electronics, and cosmetics. The improvement of various products using these materials is largely due to properties including new physical structures such as spheres or tubes, increased surface area to mass ratios, and changes in surface chemistry. Nanomaterials are typically constructed from well characterized elements and compounds (e.g. carbon, metals, and metal oxides). Due to their unique characteristics, limited information concerning their transport, fate, exposure, dose, and potential effects on ecosystems can be found in the literature. Because nanomaterials can improve many of the physical characteristics of a wide range of products, their prevalence in various microenvironments will most likely increase, along with the potential ecosystem exposure and risk. Consequently, the United States Environmental Protection Agency (USEPA) views these materials as potential and emerging contaminants. Ecosystem exposure will be affected by physical and chemical properties that control nanomaterial movement through air, soil, and aquatic ecosystems, and that influence the biological/environmental interface.

Fullerenes as a class of carbon nanomaterials are used in a wide range of commercial products and are expected to be one of the major contributors in future product development (1). Most environmental studies with fullerenes to date have used solvent exchange techniques for creating fullerene suspensions of C_{60} in water (2,3). In these studies, fullerenes were dissolved in an organic solvent, the solution was dispersed in water, and the organic solvent removed by distillation. Another approach to suspending fullerenes in water is simply to stir them in buffered suspensions for extended time periods (days to months). Both aqueous and organic solvent-assisted techniques result in the formation of stable suspensions; however, colloidal fullerene aggregates formed using solvent exchange and those formed in water-only systems differ with respect to size, shape, charge, polydispersity and also in the toxic effects they elicit (5-7). Extended stirring in water without the aid of organic solvents was used in the present study to create colloidal suspensions of aqu/C_{60} and aqu/C_{70} that contained fewer preparation artifacts and that are more representative of natural environmental systems.

Aquatic environments may be particularly vulnerable to contamination of nanomaterials due to a number of factors. These factors include uncertainties concerning ingestion, bioavailability and bioaccumulation of these materials in indicator species such as crustacean zooplankton. Planktonic invertebrates are particularly important with respect to contamination of the aquatic environment because they are the bridge in the food chain between pollutants bound to suspended particulates, algae and fish (8). Organisms such as *Daphnia magna* have been widely used as indicator species for both ecosystems and human health. In addition, nauplii of anostracans such as *Thamocephalus platyurus* have been characterized for their responses to a wide range of toxins and assays have been formatted for use in commercial toxicity screening assays. Previous studies of the effects of C_{60} fullerenes on aquatic invertebrates have shown a number of significant sub-lethal effects. For example, exposure of *D. magn*a to C_{60} fullerene at concentrations of up to 5 mg/L over 21 days showed delayed molting behavior but relatively low (40%) mortality rates (9). Changes in behavior and locomotor activity for *D. magna* exposed to C_{60} fullerenes have also been reported (10,11,8). Exposure of *D. magna* to fullerene C_{60} and carbon nanotubes have resulted in the presence of dark masses in the gut of these organisms (8,11,12). The use of radiolabelled nanoparticle agglomerates has also allowed the observation of uptake and elimination kinetics under specific conditions.

This study focuses on characterization of the changes in aqu/C_{60} and aqu/C_{70} fullerene agglomeration as the result of ingestion and depuration of these materials by the indicator organism *T. platyurus*. The implications of transport and fate of these materials is also considered.

Experimental Procedures

Preparations of media and suspensions. The C_{60} (purity 99.9%) and C_{70} (purity 99.0%) fullerenes were purchased from MER Corp. (Tucson, AZ, USA). Rapidtoxkits™ were purchased from Strategic Diagnostics (Newark, Delaware, USA). All other chemicals were of reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). For fullerene suspensions used for *T. platyurus* experiment, 100 mg of C_{60} or C_{70} were added to 400 mL double de-ionized water (DDI) (>18 M Ohms-cm). The mixtures (stirred using a magnetic plate for approximately 100 days) have been previously shown to form stable suspensions

(13). For particle size characterization prior to the exposure experiments, the suspensions were allowed to settle by gravity for 1 hr and the suspensions were sampled at 2-cm below the surface.

Suspension characterization. The method used for mass quantification of aqu/C_{60} and aqu/C_{70} is described in a separate publication (13). In this quantitation method, aqu/C_{60} and aqu/C_{70} were extracted into toluene and then analyzed by high performance liquid chromatography-ultra violet detection (HPLC-UV) and high performance liquid chromatography-mass spectrometry detection (HPLC-MS). Zeta (ζ) potentials of the suspensions were monitored using a ZetaSizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). This instrument uses phase analysis light scattering (PALS) to measure the electrophoretic mobility of charged particles. The Smoluchowski equation was used to calculate ζ-potential from electrophoretic mobility. Three measurements (12 runs per measurement) were acquired from each sample. Instrument performance was verified using NIST-traceable polystyrene microsphere standards.

Changes in fullerene aggregate size were examined by dynamic light scattering (DLS) (ZetaSizer Nano ZS). Six measurements (12 runs per measurement) were acquired from each sample. The autocorrelation function was analyzed by the cumulant method to obtain the moments of the aqueous fullerene aggregate size distribution. The fluctuations of scattered light from particles can be mathematically correlated to the diffusion coefficient. The intensity average (Z-average) hydrodynamic diameter was calculated from measured diffusivities using the Stokes-Einstein equation.

Electron Microscopy. For electron microscopic analysis, the fullerenes were analyzed from water suspensions. Droplets of fullerene suspensions were placed onto copper grids coated with perforated carbon or lacey carbon films and observed using a Tecnai transmission electron microscope (TEM) (FEI, Hillboro, OR, USA). The imaged aggregates were also analyzed by energy dispersive X-ray spectroscopy for elemental composition by EDEX software. Thin section images were taken using an FEI/Philips CM-10 TEM.

***Thamnocephalus platyurus* Assays.** *T. platyurus* were hatched from cysts following the Rapidtox™ protocol. The cyst preparation was hydrated with DDI water and incubated at 25º C under 4000 lux illumination for 30 to 45 hr. After hatching, the larval suspensions

were mixed (1:1) with fullerene suspensions and incubated for 1 hr prior to addition of the colored indicator microspheres (5 μm red polystyrene). The organisms were exposed to the microspheres for 15 min prior to fixation with Lugol solution (final concentration, 1% KI, 0.5% I_2) and final observation. The procedure for exposure of the *T. platyurus* to fullerenes prior to feeding with algae was similar to the indicator microsphere experiment except that algae were added (at 100,000 cells/mL) rather than the indicator microspheres. For fullerene exposure without indicator microspheres, the organisms were exposed to aqu/C_{60} or aqu/C_{70} for 1 hr then fixed with Lugol solution prior to extraction and analysis.

The nauplii were sub-sampled at between 10 and 20 organisms per assay and the assays were run in duplicate. Organisms were observed using a stereo microscope at about 40 x magnification and images recorded using Motic Image Plus 2.0 software.

TEM and Chemical analysis of the gut contents. For *T. platyurus*, the nauplii were exposed to aqu/C_{60} or aqu/C_{70} at 69 mg/L and 62 mg/L, respectively for 1 hr. For thin section TEM analysis, after exposure to aqu/C_{60}, the *T. platyurus* were fixed in 2% glutaraldehyde then exchanged into 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4. The organisms were then dehydrated by stepwise ethanol exchange (10, 30, 50, 90, 100% ethanol) then further exchanged from propylene oxide into EPON resin. The resin was then polymerized for 48 hr at 60º C prior to thin sectioning and TEM analysis.

For chemical analysis, the organisms were washed 3 times with DDI water by centrifugation. After centrifugation, the supernatant was removed each time and the pellet re-suspended in DDI water. For the sample blank, aqu/C_{60} was added after the control organisms were fixed to account for the non-ingested particles that were carried over in the washes. The pelleted organisms were frozen, lyophilized, extracted for 30 min with toluene on an orbital shaker, and analyzed by HPLC-MS (13).

Results and Discussion

Aqu C_{60} and Aqu/C_{70} Fullerene Preparations. The Z-average diameters for the aqu/C_{60} and aqu/C_{70} aggregates prior to feeding and without sonication showed size distribution averages of 517 ± 21 nm and 656 ± 39 nm (mean ± 95% confidence limits), respectively. Zeta (ζ) potentials for the aqu/C_{60} and aqu/C_{70} suspensions were -27.5 ± 0.7 mV and -47.5 ± 5.4 mV, respectively. Generally, colloidal suspensions of particles with absolute zeta

potentials greater than 30 mV will be stable and will not further aggregate or precipitate (14). Similar to the results by DLS, analysis of the vortexed aqu/C_{60} suspensions by transmission electron microscopy (TEM) showed aggregates that ranged from about 50 to 500 nm in length (SI Figure S1A,B).

Exposure of *T. platyurus* to Fullerene Suspensions. The acute toxicity of *T. platyurus* exposed to fullerene suspensions was evaluated using a feeding inhibition assay (Rapidtox™). Due in part to the relative stability of *T. platyurus* cysts and their ability to hatch 'on demand' under laboratory conditions, this anastracan species has been adapted and used routinely in acute toxicity screening assays (15). *T. platyurus* typically grow at a rate of 154% (in length) / day on a diet of primarily live algae (16).

In the absence of an added food source, *T. platyurus* readily accumulate indicator microspheres (5 μm) after hatching (about 30-40 hr). The assumption for this type of assay is that chemically-stressed organisms ingest these colored polystyrene microspheres at a lower rate than control organisms (17,18). The inhibition of microsphere ingestion can also be measured as a lower number of organisms that contain indicator microspheres in a given population after a specified exposure time. For this assay model, it has been previously reported that *T. platyurus* respond to toxins such as phenol in a reproducible and concentration dependent manner (19). The EC-50 that we measured for phenol used as a positive toxic control was 10 mg/L and compared closely to a previously reported value (8.3 mg/L) for this compound (19).

Because *T. platyurus* are semi-transparent organisms, exposure and accumulation of these red indicator beads could be visualized as a red opaque outline of the gut cavity (seen in light gray in Figure 1A). When *T. platyurus* were exposed to aqu/C_{60} or aqu/C_{70} fullerenes prior to exposure to the indicator beads, they accumulated the fullerenes as dark masses in their digestive tract (Figure 1B). This observed response was unusual because the uptake of indicator beads by this organism is typically inhibited by a chemically-induced stress response. The fullerenes appeared to compete with the indicator microspheres with respect to accumulation in the gut. Although some of the organisms' digestive tracks contained only red microspheres (seen as gray) or fullerenes (seen as black), many of them showed segments of each (Figure 1B). After exposure of *T. platyurus* to aqu/C_{60} or aqu/C_{70} fullerenes with no addition of indicator microspheres, the

dark masses visible in the digestive tract also appeared to be segmented (with intervening light areas) indicative of the formation of excretion pellets (Figure 1C).

The percentage of organisms accumulating the dark masses (in at least 20% of the gut tract length) was concentration dependent for both aqu/C_{60} and aqu/C_{70} fullerenes (Figure 2). Although a significant number of organisms accumulated the fullerenes at the lower concentrations tested, about 50% of the organisms accumulated observable fullerene masses in their gut at about 10 mg/L.

When the *T. platyurus* were exposed to fullerenes followed by unicellular algae, they showed a pattern of competition similar to the indicator microspheres. The ingested algae could also be observed as green bands between the dark masses of aqu/C_{60} or aqu/C_{70} fullerenes, similar to the results observed with the red indicator spheres. The fullerenes also competed in a concentration-dependent manner with the algae that accumulated in the gut (data not shown).

Structure and composition of dark masses in the gut. Electron micrographs of the transverse cross-sections of *T. platyurus* exposed to aqu/C_{60} fullerenes and polystyrene beads show the microvilli-lined lumen of the mid-gut containing aqu/C_{60} agglomerates (Figure 3 A,B). Compared to the nanomaterial aggregates used for the exposure experiments (50-500 nm), these aqu/C_{60} agglomerates appeared to be significantly larger (5-10 μm) and more angular in structure. These larger agglomerates observed in the gut were presumably assembled from smaller aggregates that had been ingested by the organisms. The micrograph in Figure 3B shows one of these agglomerates next to one of the polystyrene microspheres. Part of this agglomerate appears to have coalesced into a larger and possibly more stable structure. The composition of material that accumulated in the gut of *T. platyurus* was confirmed as aqu/C_{60} or aqu/C_{70} fullerenes by extraction and analysis by HPLC-MS. For analysis of the gut contents, the aqu/C_{60} or aqu/C_{70} fullerene-exposed organisms were subjected to fixative (Lugol's solution), washed twice with DDI water by centrifugation, lyophilized and extracted using toluene. The amount of aqu/C_{60} or aqu/C_{70} measured per organism was 87 ng and 345 ng, respectively (SI Table S1). The extraction blank in which aqu/C_{60} was added after the organisms were fixed to control for the non-ingested matrix carry-over resulted in a value of 5 ng/ organism. It is unclear why the organisms accumulated aqu/C_{70} to a greater extent (mass per organism) than aqu/C_{60};

however, the dark masses observed in the guts of the aqu/C_{70} exposed organisms were also darker and more clearly defined as compared to those exposed to aqu/C_{60} at similar concentrations.

Exposure of *T. platyurus* to aqu/C_{60} for several hours resulted in the formation of excretion pellets that settled to the bottom of the incubation wells. These pellets were removed and stored for one month (at room temperature) prior to analysis. Analysis of this material by light microscopy showed a population of agglomerates ranging in size from about 10 μm to 70 μm (Figure 4A). Some smaller agglomerates were also visible. Observation of the smaller agglomerates by TEM showed the presence of micrometer sized aggregates of aqu/C_{60} that were angular and rectangular, similar to those seen in the thin sections of the aqu/C_{60}-exposed *T. platyurus* gut (Figure 4B,C). At higher magnification, the aqu/C_{60}- agglomerates from the excretion pellets that adhered to the lacy carbon membrane appeared to be composed of smaller aggregates in the 100-200 nm size range.

Changes in Aqu/C_{60} and Aqu/C_{70} fullerenes After Ingestion and Depuration. In a typical toxicity screening assay, the polystyrene indicator spheres are concentrated in the gut then disperse after passing through the organism. In the case of the aqu/C_{60} or aqu/C_{70} fullerenes, our observations indicate the formation of larger and more stable agglomerates that form in the process of uptake, accumulation and depuration. Table 1 shows visible microscopy and electron microscopy observations as well as size distributions for the fullerene agglomerates in suspension before and after ingestion by *T. platyurus*.

The aqu/C_{60} aggregates used in the exposure experiments were in the 500-600 nm size range. These stable aggregates remained in suspension and were accumulated by *T. platyurus*. In the mid-gut, these aggregates appeared to coalesce into larger agglomerates that were relatively angular in appearance and were in the 5-10 μm size range. Upon depuration, and storage these agglomerates appeared (by light microscopy) to form stable agglomerates in the 10–70 μm size range; however, observation by TEM indicated that these agglomerates were composed of smaller aggregates in the 100-200 nm size range. In order to obtain comparative size distribution measurements between the pre-exposure and post-exposure materials by DLS, both samples of aqu/C_{60} were sonicated for 10 min. Following sonication, there was no significant difference between the two treatments (256

± 14 nm for pre-exposure and 273 ± 16 nm post-depuration). HPLC-MS analysis also indicated no significant alteration of the fullerenes after passing through the gut.

Aquatic crustacean filter-feeders (such as *T. platyurus*) typically feed on unicellular algae in the micrometer size range. These organisms, however, can be grown in the laboratory on diets ranging from bacteria to baker's yeast. In addition, organisms such as *D. cucullae* have been shown to accumulate polystyrene beads in the 5–35 μm size range (20). Prior to ingestion the fullerene agglomerates were relatively small (approximately 500 nm), stable and remained in suspension. Nevertheless, the *T. platyurus* accumulated these aggregates similarly to the larger (5μm) indicator beads.

Although the filtration rate of aquatic crustaceans has been reported to be affected by the size and viability of food organisms (21) and the preferred size range for food particles for these organisms is > 1 μm, *D. magna* have been shown to ingest and accumulate agglomerates of C_{60} at sizes below 0.45 μm (8). *D. magna* have also been shown to ingest and accumulate carbon nanotubes with an average length of < 200 nm (22). These authors also reported that the nanotubes aggregated in the gut and were not efficiently eliminated without continuous exposure to dispersed nanotubes or additional algae were available in the media. In another experiment reported by Roberts et al. (11), lipid-coated nanotubes were ingested by *D. magna* and after accumulation in the gut, the nanotubes were reported to have been excreted by the organisms as aggregated material that was stripped of the original lipid coating that allowed them to remain dispersed in suspension (22).

Filter-feeding organisms such as *T. platyurus* and *D. magna* play important roles in the ecosystem due to their rapid filtration rate and have been shown to have impacts on water turbidity and algal composition (23). In addition, Filella et al. recently illustrated the potential for *Daphnia sp.* to impact the size distribution of inorganic colloids, but also note that this effect would be dependent on their population density (24). Because of the tendency for these organisms to filter and excrete carbon-based nanomaterials in an agglomerated state, they have the potential to significantly change the size distribution and fate of these nanoparticles in the environment. Others have investigated the effects of natural organic matter, and water chemistry on particle size and stability in natural waters, and the resulting effects to fate and transport (25-27). Because this study illustrates that

filter-feeding organisms have the potential to influence particle agglomerate size resulting in impacts to the material's fate and transport in the environment, the biological structure of ecosystems should also be considered along with the physical and chemical properties of natural waters when predicting the environmental fate, transport, and exposure potential of carbon-based nanomaterials.

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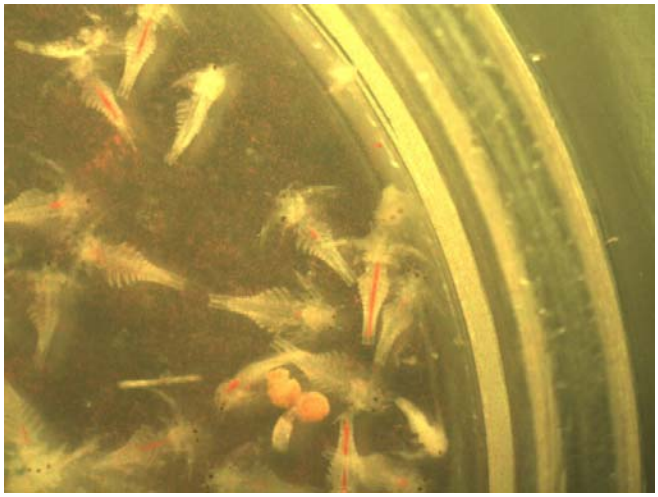
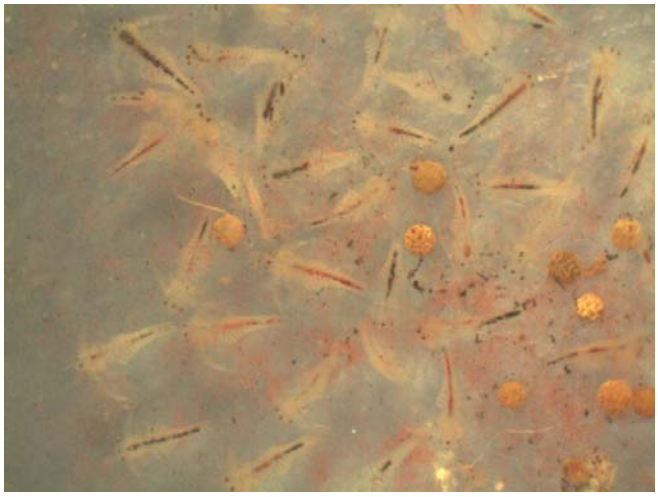
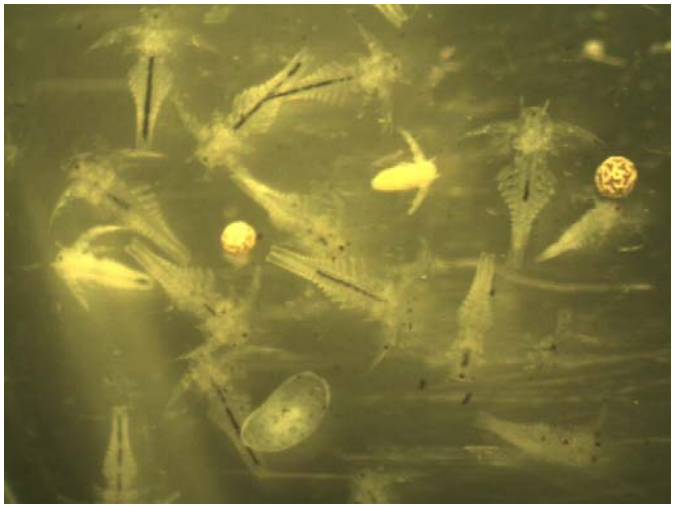


A



B



C

Figure 1 A, B, C

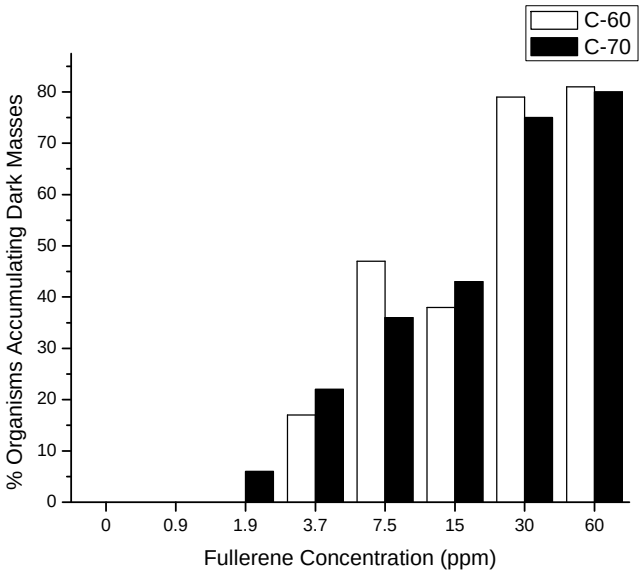
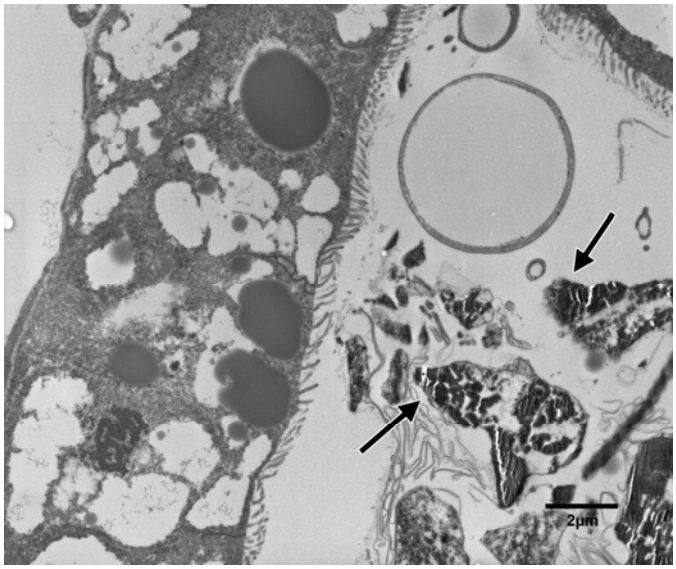
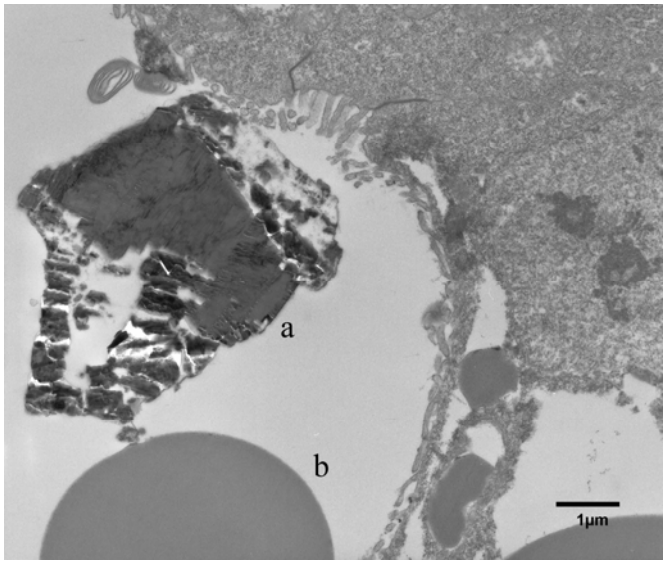

C-60
C-70
% Organisms Accumulating Dark Masses
0
10
20
30
40
50
60
70
80
0
0.9
1.9
3.7
7.5
15
30
60
Fullerene Concentration (ppm)

Fig. 2

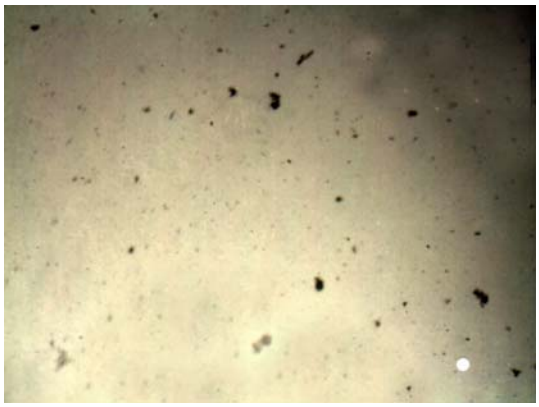


A

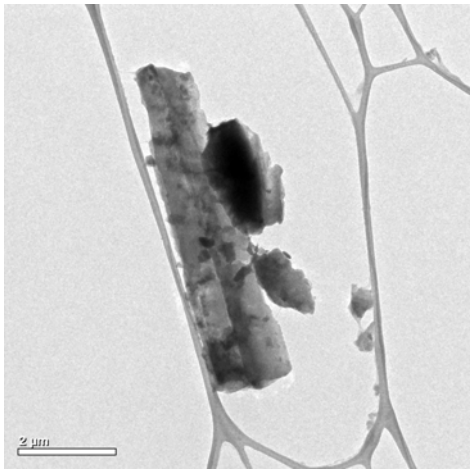


B

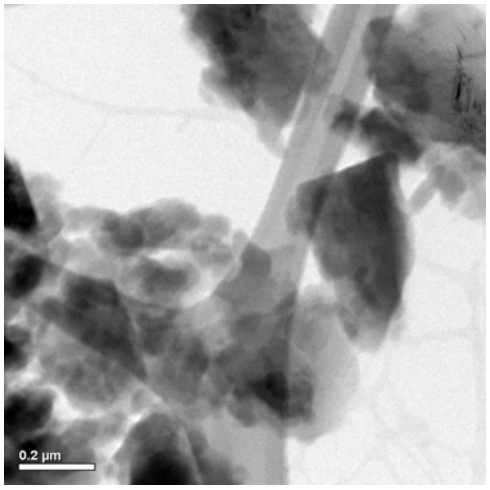
Figure 3 A, B



A



B



C

Fig. 4 A, B, C

Table 1 Changes in fullerenes after ingestion, accumulation and depuration by *T. platyurus*.

Sample	Agglomerate size by Microscopy	Z average size	Z potential (mV)
Aqu/C_{60} prior to feeding	50-500 nm	517 +/-20	-27.5
Aqu/C_{60} prior to feeding; after sonication	ND	256 +/-14	-35.1
Aqu/C_{60} mid-gut thin section TEM	5-10 μm	ND	ND
Aqu/C_{60} after excretion prior to sonication	10-70 μm*	ND	ND
C_{60} after feeding after sonication	ND	273 +/-16	-35.3
C_{70} prior to feeding	50-500 nm	657 +/-37	-47.5

*100-200nm sub-structure

Figure Legends

Figure 1 Accumulation of aqu/C_{70} fullerene and red (observed as gray) polystyrene indicator beads by *T. platyurus*. (A) Control organisms, 30-45 hr larvae exposed to indicator beads only; (B) Larvae exposed to aqu/C_{70} (60 mg/L) for 60 min prior to addition of indicator beads; (C) Larvae exposed to aqu/C_{70} only, 60 mg/mL, 60 min.

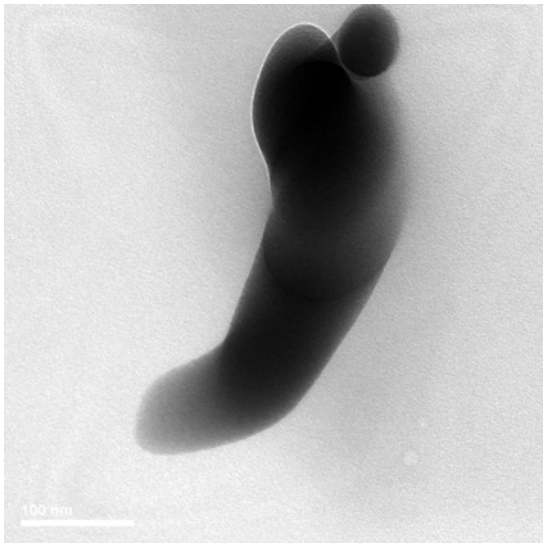
Figure 2 Uptake of aqu/C_{60} and aqu/C_{70} fullerenes by *T. platyurus*. Bars represent % of organisms showing greater than 20% gut length occupied by accumulated fullerenes. Exposure conditions were the same as for Fig. 2C.

Figure 3 Transmission electron micrographs of transverse thin cross-sections of *T. platyurus* after exposure to C_{60} (60 min., 60 mg/mL). (A) Arrows indicate aqu/C_{60} agglomerates (bar = 2 μm); (B) (a) aqu/C_{60} agglomerate, (b) polystyrene microsphere (bar =1 μm).

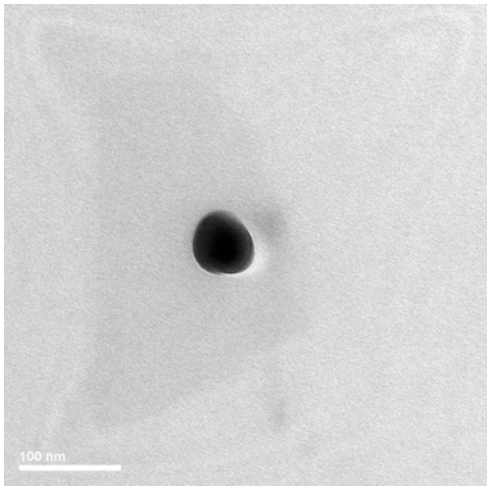
Figure 4 Light microscopy and TEM from *T. platyurus* after exposure to aqu/C_{60}. (A) Light microscopy image of excretion pellets after 30 days of incubation (white circle = 70 μm). (B) Relatively large angular agglomerates adhering to lacy carbon film (bar = 2μm); (C) Agglomerate composed of smaller aqu/C_{60} aggregates (bar = 0.2 μm).

Supplemental Figure Legends

Figure S1 Transmission electron micrographs of aqu/C_{60} fullerene aggregates prior to ingestion by *T. platyurus*. Selected images were representative of observations. (A) Larger particle; (B) Smaller particle, (bars = 100 nm).



A



B

Figure S1 A,B

Table S1 Identification and quantitation of fullerenes accumulated in *T. platyurus* gut.

Sample	**Total mass in extract by LC (ng/50 organisms)**	**Mass (ng/organism)**
C_{60}	1625	27
C_{70}	17,503	345
Blank for C_{60}	262	ND