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Fullerene Exposures with Oysters: Embryonic, Adult, and Cellular Responses

AMY H. RINGWOOD*
Department of Biology, University of North Carolina at Charlotte, 9201 University City Boulevard, Charlotte, North Carolina 28223

NICOLE LEVI-POLYACHENKO
Department of Plastic and Reconstructive Surgery, Wake Forest University Health Sciences, Winston-Salem, North Carolina 27157

DAVID L. CARROLL
Center for Nanotechnology and Molecular Materials and Department of Physics, Wake Forest University, Winston-Salem, North Carolina 27105

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Oysters are an ecologically important group of filter-feeders, and a valuable toxicology model for characterizing the potential impacts of nanoparticles to marine organisms. Fullerene (C60) exposure studies with oysters, *Crassostrea virginica*, were conducted with a variety of biological levels, e.g., developmental studies with embryos, whole organism exposures with adults, and isolated hepatopancreas cells. Significant effects on embryonic development and lysosomal destabilization were observed at concentrations as low as 10 ppb. Moreover, based on our extensive experience with the lysosomal assay, the lysosomal destabilization rates at fullerene concentrations ≥100 ppb were regarded as biologically significant, as they are associated with reproductive failure. Interestingly, there was no significant increase in lipid peroxidation levels in hepatopancreas tissues. Oyster hepatopancreas tissues are composed of lysosomal rich cells, and confocal microscopy studies indicated that the fullerene particles readily accumulated inside hepatopancreas cells within 4 h. Fullerene aggregates tended to be localized and concentrated into lysosomes. The microscopic work in conjunction with the lysosomal function assays supports the premise that endocytotic and lysosomal pathways may be major targets of fullerenes and other nanoparticles. Nanoparticles that affect normal lysosomal and autophagic processes may contribute to long-term, chronic problems for individual health as well as ecosystem health.

Introduction

The development of manufactured materials that incorporate nanoparticles presents exciting potential for innovative products that will certainly affect all aspects of our society. Numerous products are currently marketed and more are rapidly emerging (*1*). While many will certainly lead to significant innovations in medical applications and environmental technologies, there are potential environmental as well as human health risks of engineered nanoparticles that are not yet well-characterized or understood (*2*−*5*). Nanoparticles will be introduced into aquatic environments during production processes and also as a result of release following their use in electronic and biological applications. Carbon nanoparticles, fullerenes and nanotubes, are already produced in large quantities (greater than 1500 tons per year), are used in numerous consumer products, and have the potential to become a significant environmental contaminant (*6, 7*). Fullerene C60 (Buckminsterfullerenes) are spherical molecules composed of sixty carbon atoms with unique multifunctional properties that make them especially valuable in various optics and imaging products, and a variety of biomedical applications (*8*−*11*). While fullerene molecules are only one nanometer diameter in their pure form, they do aggregate in aqueous media to form a variety of size and shape configurations (*12*).

Fullerene (C60) exposure studies with oysters, *Crassostrea virginica*, were conducted with a variety of biological levels, e.g., developmental studies with embryos, whole organism exposures with adults, and isolated hepatopancreas cells. Oysters and other bivalve mollusks are excellent model organisms for characterizing potential nanoparticle impacts on aquatic ecosystems. As filter-feeding organisms, oysters spend their lives filtering large volumes of water (hundreds of liters/day) processing microalgae, bacteria, sediments, particulates, and natural nanoparticles. Oysters and mussels have long been recognized as valuable pollution bioindicators. This extensive background of information based on pollutants and methodologies for identifying impaired biological responses are important factors that enhance the use of filter-feeding bivalves as valuable models for nanoparticle toxicity studies. Moreover, estuaries tend to be major depositional areas, commonly inhabited by oysters and other filter feeders, so these studies are essential for characterizing the responses of potential target receptors to environmental inputs of nanoparticles.

Lysosomal destabilization and lipid peroxidation assays were used to identify toxic responses in adult oysters. Lysosomes are involved in many essential cellular functions, including defense, tissue repair and turnover, autophagy, and nutrition. Lysosomal function assays of hepatic or blood cells have long been regarded as valuable indicators of pollutant-induced injury (*13*). Neutral red techniques for assessing lysosomal destabilization have been used successfully in fish and invertebrate taxa throughout the world for more than a decade, and there is a substantial body of literature validating that environmental pollutants cause destabilization of lysosomes (*14*−*18*). From a broad range of studies conducted with oysters, laboratory and field, we have consistently shown that (1) pollutants and various toxins cause increases in lysosomal destabilization, (2) lysosomal responses were significantly correlated with sediment and tissue contaminants in a dose-dependent manner, and (3) normal embryonic development is very low when lysosomal destabilization rates of parents are significantly elevated (*15, 19*−*22*). Lipid peroxidation is an indicator of damage to cellular lipids, especially cell membranes, that occurs when free radicals (e.g., OH•) react with lipids. Moreover, the free radical-induced damage propagates additional cytotoxic products that can damage DNA and enzymes (*23, 24*). Increased lipid peroxidation has been demonstrated in response to contaminant exposures in fish and bivalves (*25, 26*), and has been proposed as a potentially major mechanism involved in the mediation of nanoparticle toxicity

* Corresponding author phone: (704) 687-8501; fax: (704) 687-3128; e-mail: ahringwo@uncc.edu.

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as well as the associated solvents used for nanoparticle preparations (*27*−*30*). Finally, an important question regarding nanoparticle toxicity is whether the nanoparticles actually enter and accumulate in cells, or if individual or aggregates of nanoparticles adhere to the surface of cells, impairing transport or other essential functions that could contribute to toxicity. To address this issue, we report here on confocal microscopy studies to verify the accumulation of fullerenes by hepatopancreas cells.

Many aquatic organisms have complex life histories that involve external fertilization and embryo development, and various larval forms. It is essential that nanoparticle assessment strategies consider relative toxicities of different life history stages. Are embryonic and larval forms more sensitive than adults? Oyster embryology has been well characterized, and oyster embryo assays have frequently been used to evaluate the potential toxicity of toxins, using control laboratory exposure studies as well as water and sediment samples from natural environments. Therefore, the results of embryo toxicity assays following exposures to fullerene concentrations similar to those used for the adult studies are presented here to address the question of relative sensitivity of the different assessment methods with oyster embryos, adult oysters, and isolated hepatopancreas cells.

Experimental Section

Synthesis and Characterization of Fullerene "C60" Suspensions. Stable aqueous "C60" suspensions were prepared in toluene using previously published procedures (*31*). Briefly, fullerene "C60" suspensions (0.5 mg of C60 from Sigma Chemical per mL of toluene) were sonicated for 10 min in a water bath (Branson) until a uniform purple solution was obtained and all C60 had been dissolved. Following sonication in toluene, an equal volume of deionized water was added to the toluene/"C60" suspension and an organic/water phase separation was observed. This solution was sonicated in a water bath until all the toluene evaporated, and was filtered through a 0.2 μm filter. Fullerene suspensions were characterized by spectral methods to confirm the presence of suspended, colloidal material, as well as determine concentrations of material in solution. Characteristic absorption peaks occur at 265 and 356 nm. The results of dynamic light scattering studies indicated that the particle sizes in the toluene suspension ranged from 10 to 100 nm, with a predominance of 33 nm particles. For the final aqueous solutions, the average particle size was 122 nm, indicating some aggregation of the C60 molecules dispersed in water. Blank solutions (e.g., analogous toluene water suspensions with no fullerenes added) were processed by sonication and evaporation, and tested for the various assays at the highest possible concentration that would be used in the assays; no significant differences from seawater-only controls were observed. NMR analysis of the fullerene preparations verified that there was no residual toluene.

Dynamic light scattering analyses (Zetasizer Nano-S, Malvern Instruments, Southboro, MA) of seawater preparations of the different exposure concentrations were conducted. These types of analyses are somewhat problematic because natural seawater has a high background level of nanoparticles (including organics and natural colloids, viral DNA, etc.), so distinguishing engineered nanoparticles from natural nanoparticles can be difficult. Based only on those peaks that did not co-occur with background peaks, the particle size appeared to be the smallest for 10 ppb solution (approximately 150 nm), around 200 nm for the 100 ppb solution, and the largest particle size was observed for the highest concentration (1000 ppb, e.g., a heterogeneous range of sizes, mostly spanning 500−900 nm). These data do suggest that particle size can be concentration dependent, as well as being affected by organic matter and naturally occurring nanoparticles, and recent studies have demonstrated that these natural components may serve to minimize agglomeration of nanoparticles (*32*).

Oyster Hepatopancreas Studies. Adult oysters were exposed to a range of fullerene concentrations, nominal concentrations of 1−500 ppb (μg/L), in natural seawater (3−4 small adult oysters per liter of 25 ‰ 0.45 μm filtered natural seawater) for 4 days; oysters were fed cultured phytoplankton (*Isochrysis galbana*) daily; and results are presented from 2 replicate experiments. At the end of the exposure period, the oysters were shucked and the tissues were rinsed with filtered seawater. A small piece of hepatopancreas tissue was removed and processed immediately for the lysosomal destabilization assay, and subsamples of hepatopancreas tissues were frozen at −80 °C for lipid peroxidation analyses.

Primary cell preparations of hepatopancreas cells from individual oysters were generated as described below using calcium-magnesium-free saline (CMFS) for the initial stages of the lysosomal destabilization assays. The cells were resuspended in a range of fullerene concentrations prepared in 1:1 CMFS: 0.22 μm filtered natural seawater, 25‰ salinity. The cells were then pipetted onto glass microscope slides in glass Petri dishes (2 slides per oyster per concentration), and incubated at room temperature (24 °C) for 24 h. At the end of the exposure period, cells were recovered using cold CMFS and a cell scraper, rinsed in CMFS, and used for the lysosomal destabilization assay. The results of two separate, fully replicated experiments (n = 3 or 4 oysters, replicated cell preparations from separate oysters/treatment) are presented. Subsamples of the cells were fixed in buffered formalin after 4 and 24 h of exposure, and used for confocal microscopy studies to determine if fullerenes were accumulated inside the hepatopancreas cells.

Lysosomal Destabilization Assays. A neutral red assay was used to evaluate lysosomal stability in live cells derived from hepatopancreas tissues of individual bivalves (*16*). Freshly dissected tissues were minced and incubated in Ca^{2+}-Mg^{2+}-free physiological saline (CMFS) and trypsin to facilitate dissociation of the cells, and then centrifuged though fine nylon mesh to generate primary cell preparations. The cells were then incubated in CMFS containing neutral red for a fixed time period (60 min), and the numbers of cells with neutral red retained in the lysosomes (e.g., stable lysosomes) or with neutral red leaking into the cytoplasm (destabilized lysosomes) were determined with a compound microscope (600× magnification; >50 cells scored for each preparation) and the results were expressed as % destabilized lysosomes. The data were analyzed by analysis of variance (ANOVA) using Sigma Stat; normality and equal distribution of the data were verified. An *a posterior* analysis was used to identify significant differences between treatments (e.g., when $p <$ 0.05).

Lipid Peroxidation Assays. Lipid peroxidation was measured using the thiobarbituric acid (TBA) reaction for the detection of malondialdehyde (MDA), a lipid peroxidation product (*26, 33*). Hepatopancreas tissues were homogenized in potassium phosphate buffer, and reacted with TBA and butylated hydroxytoluene, and the MDA derivatives were then detected with a platereader (KC Junior) at 532 nm. Lipid peroxidation concentrations were determined from a standardized calibration curve with malondialdehyde tetraethylacetal as the standard. The data were expressed as nmol MDA/mg tissue wet weight and statistically analyzed as described above.

Confocal Microscopy. Hepatopancreas cells (control and fullerene exposed) were fixed in buffered formalin, washed in CMFS to remove excess fullerene particles, pipetted into microscope chambers, and viewed with a Zeiss LSM 510 confocal microscope. Fullerene aggregates were detected

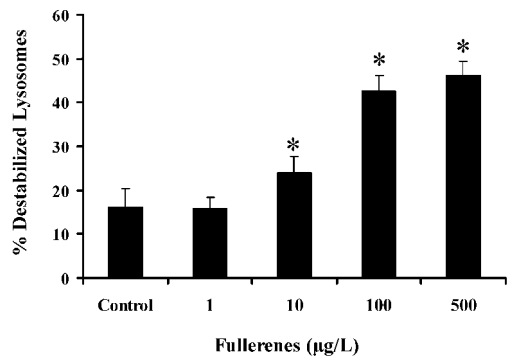

FIGURE 1. Lysosomal destabilization rates of adult oysters exposed to a range of fullerene concentrations for 4 days. Data are presented as means ± standard deviations; results are the combined data from replicated experiments. Asterisks (*) indicate significant differences from controls ($p < 0.05$).

using an excitation wavelength of 458 nm and a long pass filter wavelength of >650 nm as previously described (*31*), and the images were optically sectioned using Axiovision software to verify the intracellular location of the fullerene particles.

Oyster Embryo Assays. Oyster embryo development assays were conducted with gametes stripped from ripe female and male oysters (gametes were pooled from at least 4 individuals/sex) as previously described (*21, 34*). Newly fertilized oyster embryos (approximately 2 h postfertilization) were added to a range of fullerene concentrations (nominal concentrations of 1 ppb to 1000 ppb, 4 replicate glass tubes per treatment, 25‰ 0.45 μm filtered seawater; each replicate tube contained approximately 200 embryos in 10 mL). After 48 h, the embryos were fixed in buffered formalin, and the number of normal embryos (e.g., embryos that had reached the D-veliger larval stage, with a normal shaped shell and well-developed velum) was determined. These experiments were repeated with 3 different gamete batches, and one set was incubated entirely in the dark to determine if holding the embryos in the laboratory exposed to fluorescent lighting affected fullerene toxicity. The data were expressed as percent of controls; significant treatment effects were identified using ANOVA as described earlier, and regression analyses were conducted.

Results and Discussion

After adult oysters were exposed for 4 days to fullerenes added to seawater, significant perturbation of lysosomal function was observed (Figure 1). Statistically significant effects were observed at concentrations as low as 10 ppb, and at the higher concentrations, the results indicated that greater than 40% of the hepatopancreas cells were functionally impaired. However, there was no significant increase in lipid peroxidation levels in hepatopancreas tissues at all concentrations tested (Figure 2). The lipid peroxidation levels observed over all treatment concentrations were typical of those observed under control or reference conditions in previous field and laboratory studies (*19, 26*). These results suggest that fullerene exposures did not elicit excess oxyradical production or peroxidative damage, and probably did not contribute significantly to lysosomal dysfunction.

Fullerene exposures with primary cell preparations of isolated hepatopancreas cells yielded results similar to the whole animal exposure studies. A significant increase in lysosomal destabilization was observed at 10 ppb but not at 1 ppb, with dose dependent increases in destabilization rates with increasing fullerene concentrations (Figure 3). The background control levels of destabilization rates were somewhat higher than that observed with cells harvested in

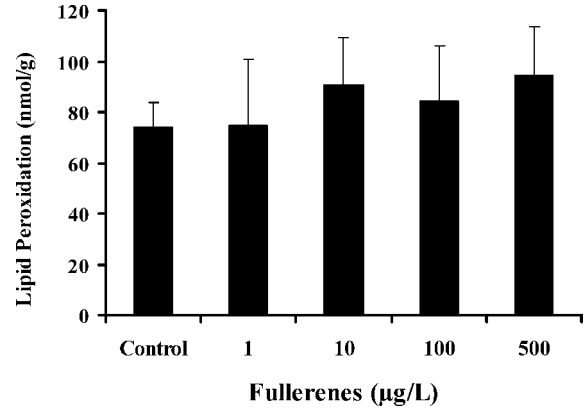

FIGURE 2. Lipid peroxidation levels (nmol/g of malondialdehyde) of adult oysters exposed to a range of fullerene concentrations for 4 days. Data are presented as means ± standard deviations; results are the combined data from replicated experiments. There were no significant differences between treatments.

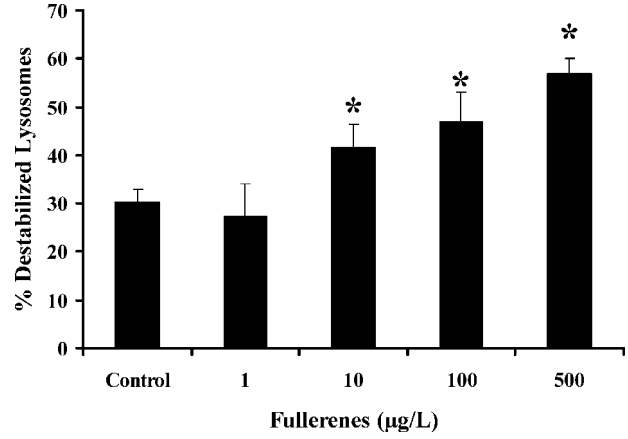

FIGURE 3. Lysosomal destabilization rates of isolated hepatopancreas cells of oysters exposed to a range of fullerene concentrations for 24 h. Data are presented as means ± standard deviations; results are the combined data from replicated experiments. Asterisks (*) indicate significant differences from controls ($p < 0.05$).

the whole oyster studies, but the general patterns of toxicity were similar. Confocal microscopy studies with subsamples of cells indicated that the fullerene particles readily accumulated inside cells within 4 h (Figure 4). Lysosomes tended to have some very low levels of autofluorescence as evidenced in the control cells, which was much different from the intensity and brightness observed with the fullerene-exposed cells. While only moderate increases in fluorescence were observed with hepatopancreas cells exposed to 10 ppb, cells exposed to 100 ppb fullerenes were characterized by intense fluorescence, distinctly compartmentalized. Based on our direct observations of the cells and careful evaluations of the light micrographs through the confocal cell sections, as well as our extensive experience with these cells, the fullerene particles tended to be localized and concentrated into lysosomes, and there was no evidence of fullerene particles in cell nuclei. These studies were conducted with nonadherent cells that were fixed in buffered formalin just prior to analyses, and were not stained with any vital stains or organelle marker dyes, so we can not say that fullerenes were not associated with other organelles such as mitochrondria. However, these are the first studies to indicate

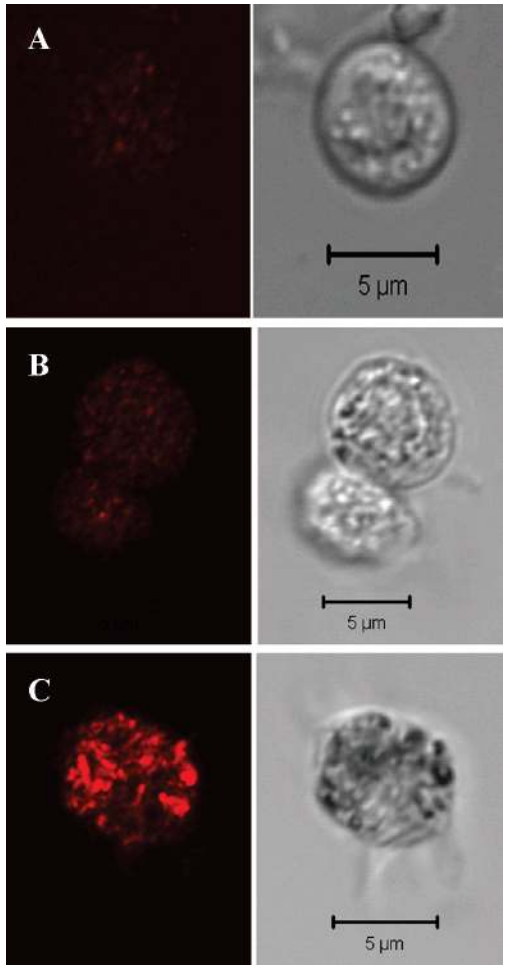

FIGURE 4. Confocal images of isolated oyster hepatopancreas cells. The top frames (A) are fluorescent and light micrograph images of a representative control cell, while frames (B) and (C) are fluorescent and light micrograph images of representative cells exposed to 10 and 100 ppb fullerenes for 4 h. In the fullerene exposed cells, the nanoparticles appeared to be concentrated in lysosomes.

that fullerenes do pass through oyster hepatopancreas cell membranes, and lysosomes do appear to be at least one of the major sites of concentration.

Oyster embryos were not more sensitive than adult oysters, and showed evidence of toxicity at similar concentrations. No toxicity was observed at the lowest concentration (1 ppb) where embryo development was approximately 98% of controls. However, as with adult oysters and isolated hepatopancreas cells, significant adverse effects on embryo development were observed at the 10 ppb fullerene exposure, and there was a highly significant dose dependent decrease in the percentage of normal embryos with increasing fullerene concentrations (Figure 5). There were no differences between the assays conducted in the dark or under ambient fluorescent light conditions.

Endocytotic pathways are likely means of nanoparticle transport, into epithelial cells from environmental contact or movement between tissues, cells, and organelles (*35*−*37*). Nanoparticles may be transferred to lysosomes following endosomal phagocytosis, or by microphagy of intracellular nanoparticles that enter by diffusion, so lysosomes are a likely intracellular target for nanoparticle deposition and toxicity (*4*). One of the cellular damage biomarkers used for the studies presented in this paper was the lysosomal destabilization assay, conducted with hepatopancreas cells. Hepatopancreas tissues in oysters play significant roles in nutrient utilization,

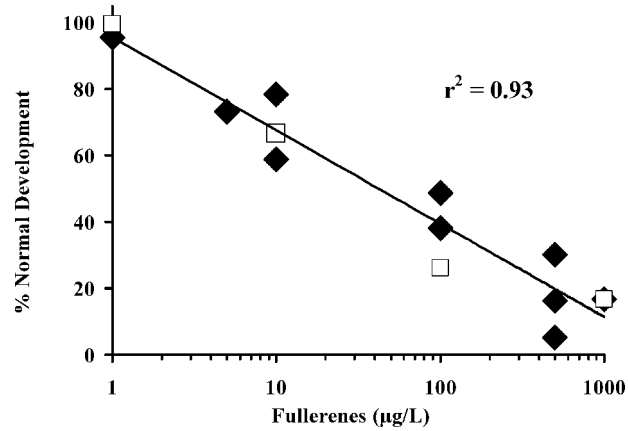

FIGURE 5. Effects of fullerene exposures on embryonic development. Separate symbols for the different concentrations represent experiments with 3 different batches of embryos; and each data point represents 4 replicate tubes of embryos for each treatment, approximately 200 embryos/tube. The open squares indicate an experiment in which the exposure tubes were held in the dark. Treatments ≥10 ppb were significantly different from the controls, and there was a highly significant dose response ($p < 0.01$), with the regression coefficient as shown in the figure.

and are also a primary site of toxin accumulation and detoxification, similar to hepatic tissues of any organism. The lysosomal destabilization assay is a valuable sensitive indicator of cellular and physiological toxicity that was used here to demonstrate the toxicity of fullerenes in oyster hepatopancreas cells.

In toxicological studies, it is important to consider the biological significance of a toxicity end point. While statistically significant differences were observed in hepatopancreas cells from adults exposed to fullerenes at 10 ppb, these may not be biologically significant effects. At this concentration, the mean lysosomal destabilization rate was less than 30%. Based on a range of field as well as laboratory studies, the levels of destabilization observed in oysters exposed to the 10 ppb treatment were within a normal range expected for control or reference oysters. Lysosomal destabilization under nonperturbed conditions can range between 15 and 30%, which is believed to reflect normal cell turnover in these tissues and are not associated with more serious adverse effects. In these studies laboratory control oysters tended to have very low levels of destabilization, and while the 10 ppb treatment did elicit a significant increase, the significance to the overall fitness of the oysters is uncertain. On the other hand, exposure to concentrations of 100 ppb fullerenes resulted in lysosomal destabilization rates that exceeded 40%. From previous studies, we have demonstrated that lysosomal destabilization rates that are around 40% and higher are associated with reproductive failure. The gonadal tissues of oysters are closely associated with the hepatopancreas tissues, where significant nutrient transfer is essential for the production of the gametes. Gamete viability of embryos derived from parents with high lysosomal destabilization rates is severely affected so that normal development rates are reduced to extremely low levels (<10%) (*21*). Therefore, adult oysters exposed to fullerene inputs greater than 10 ppb could experience adverse effects on reproductive success as well as other cellular and physiological responses. However, it is important to remember that as filter feeders, oysters are likely to accumulate the nanoparticles over longer periods, so adverse effects could be realized at lower environmental concentrations.

Similar levels of sensitivity were observed at all levels of investigation: whole animal exposures, primary cell expo-

sures, and embryo exposures. The concentrations of fullerenes used in these investigations were fairly low, in an effort to consider environmentally relevant regimes. There were no adverse effects observed with the 1 ppb treatment, suggesting that levels below this would not be expected to be problematic for either adult or embryonic oysters, or similarly sensitive species. Some statistically significant changes were observed at the 10 ppb exposures, and exposures above this concentration caused biologically significant toxic effects. There are few studies with marine organisms, but the concentrations at which toxic effects were observed in these studies are lower than many of those reported for fullerene studies with freshwater invertebrates reviewed in Klaine et al. 2008 (*7*). As expected, toxicity based on sublethal responses such as those used in our studies occurs at lower concentrations than studies based on lethality. For example, moderate or no adverse effects on zebrafish embryonic development were observed at fullerene exposures of 100 ppb, but at the 200 ppb exposures, toxicity increased dramatically (*38*). Toxicity has been observed with various systems, including cultured mammalian cells, fish, and fish embryos (*27, 28, 38*), while relatively low toxicities have been reported in others (*39, 40*). There is an ongoing debate about the differential toxicity of fullerene preparations, especially with regard to the use of tetrohydrofuran (THF) as a solvent. There is growing evidence that THF can contribute significantly to lipid peroxidation and or other indicators of toxicity (*41, 42*). No solvent-related toxicities were observed with the C60 toluene preparations in our studies.

Although we did not conduct more comprehensive analyses of other subcellular compartments, the microscopic work in conjunction with the lysosomal function assays supports the premise that endocytotic and lysosomal pathways may be major targets of fullerenes and other nanoparticles. These results are consistent with previous oyster studies conducted with quantum dots (*43*) and recent studies with mussels (*44*) as well as mammalian models (*37*). There are important questions with regard to how shape or size affects the tendency for nanoparticles to accumulate via endocytosis or perturb lysosomal function that need to be addressed. In all organisms, lysosomes play critical roles in autophagic processes (removing and recycling of damaged organelles and other cellular components), nutrient processing in hepatic cells, and developmental processes. Impairment of these processes is associated with a variety of degenerative diseases and pathologies (*45, 46*). Oyster hepatopancreas cells are lysosomal rich cells that can be used as valuable models for characterizing the potential impacts of nanoparticles on fundamental cellular processes associated with health and fitness in humans and other species as well as aquatic organisms. Nanoparticles that accumulate in lysosomes or affect normal lysosomal and autophagic processes may contribute to long-term, chronic health problems for individual health as well as ecosystem health. As an emerging material, there are a lot of unknowns with regard to the form "C60" pollution will take or the magnitude of exposures, but these studies indicate that significant inputs to estuarine environments should be carefully evaluated to avoid significant sublethal impacts on oysters and other biota.

Acknowledgments

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