Manufactured Nanomaterials (Fullerenes, C_{60}) Induce Oxidative Stress in the Brain of Juvenile Largemouth Bass

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Although nanotechnology has vast potential in uses such as fuel cells, microreactors, drug delivery devices, and personal care products, it is prudent to determine possible toxicity of nanotechnology-derived products before widespread use. It is likely that nanomaterials can affect wildlife if they are accidentally released into the environment. The fullerenes are one type of manufactured nanoparticle that is being produced by tons each year, and initially uncoated fullerenes can be modified with biocompatible coatings. Fullerenes are lipophilic and localize into lipid-rich regions such as cell membranes *in vitro*, and they are redox active. Other nano-sized particles and soluble metals have been shown to selectively translocate into the brain via the olfactory bulb in mammals and fish. Fullerenes (C_{60}) can form aqueous suspended colloids (nC_{60}); the question arises of whether a redox-active, lipophilic molecule could cause oxidative damage in an aquatic species. The goal of this study was to investigate oxyradical-induced lipid and protein damage, as well as impacts on total glutathione (GSH) levels, in largemouth bass exposed to nC_{60}. Significant lipid peroxidation was found in brains of largemouth bass after 48 hr of exposure to 0.5 ppm uncoated nC_{60}. GSH was also marginally depleted in gills of fish, and nC_{60} increased water clarity, possibly due to bactericidal activity. This is the first study showing that uncoated fullerenes can cause oxidative damage and depletion of GSH *in vivo* in an aquatic species. Further research needs to be done to evaluate the potential toxicity of manufactured nanomaterials, especially with respect to translocation into the brain. *Key words:* antioxidant defense system, fish, fullerenes, glutathione, lipid peroxidation, manufactured nanomaterials, toxicity. *Environ Health Perspect* 112:1058–1062 (2004). doi:10.1289/ehp.7021 available via *http://dx.doi.org/* [Online 7 April 2004]

Nanomaterials are defined by the U.S. National Nanotechnology Initiative as materials that have at least one dimension in the 1- to 100-nm range. Nano-sized materials are naturally present from forest fires and volcanoes, viral particles, biogenic magnetite, and even protein molecules such as ferritin. Recently, anthropogenic sources have also produced nano-sized materials—unintentionally from combustion by-products and intentionally as manufactured nanomaterials. Engineered nanomaterials are useful because of their large surface area:mass ratio, which makes them important as catalysts in chemical reactions, and they have desirable properties as drug delivery devices, as imaging agents in medicine, and in consumer products such as sunscreens and cosmetics (Colvin 2003).

The aquatic environment may be contaminated from consumer products (e.g., sunscreens and cosmetics), as well as spillage from manufacturing and shipping. It is unknown at what quantities these nanomaterials may be found in the environment, and it is especially difficult to predict as the number of products that use nanomaterials increases. As a comparison, lipophilic chemicals such as polycyclic aromatic hydrocarbons can be found at up to 4 ppm in produced water [International Association of Oil & Gas Producers (OGP) 2002], although steady-state concentrations are usually in the low ppb range and below [OGP 2002; San Francisco Estuary Institute (SFEI) 2003]. Because fullerenes are being produced by the ton (Colvin 2003), it is likely that they will eventually be found in the environment at measurable concentrations. Fullerenes can also be coated at the time of production with a variety of biocompatible materials, but it is unknown how long those coatings will stay on the fullerenes during weathering in an environmental setting, and what will happen to the fullerenes once the coating is removed. The likelihood of coating breakdown has been shown in cell culture systems, where quantum dots with cadmium-selenium cores were initially rendered nontoxic with coatings, but if the quantum dots were either exposed to air or ultraviolet radiation for as little as 30 min, they became extremely cytotoxic (Derfus et al. 2004). Therefore, this study was designed to evaluate the toxicity of uncoated fullerenes to an environmentally relevant species, the largemouth bass.

There are three areas of primary concern in terms of toxicity of fullerenes and engineered nanomaterials: *a*) some manufactured nanomaterials (especially the fullerenes) are engineered to be redox active (Colvin 2003); *b*) nano-sized particles partition into cell membranes and especially mitochondria both *in vivo* and *in vitro* (DeLorenzo 1970; Foley et al. 2002; Li et al. 2003); and *c*) research on nano-sized particles in mammalian systems shows that there is a selective transport mechanism from the olfactory nerve into the olfactory bulb (Bodian and Howe 1941; DeLorenzo 1970; Howe and Bodian 1941; Oberdörster et al. 2004). This pathway also exists in rodents and fish for soluble metals (Tjälve and Henriksson 1999; Tjälve et al. 1995).

I hypothesized that this neuronal translocation pathway could also exist in fish for redox-active, lipophilic fullerenes, causing oxidative damage in the brain. I show here that juvenile largemouth bass exposed to 0.5-ppm aqueous uncoated fullerenes (nC_{60}) for 48 hr had a significant increase in lipid peroxidation of the brain, and glutathione (GSH) depletion in the gill.

Materials and Methods

Fullerenes. Uncoated 99.5% pure fullerenes (SES, Houston, TX) were water solubilized using standard methods (Deguchi et al. 2001) by the Center for Biological and Environmental Nanotechnology, Rice University (Houston, TX) and were a generous gift for this study. Briefly, fullerenes (100 mg/L) were dissolved in tetrahydrofuran (THF), sparged with nitrogen, stirred overnight in the dark, and filtered through a 0.22-µm nylon Osmonics filter (GE Water Technologies, Fairfield, CT); MilliQ water (Milliport Corp., Bedford, MA) was added to an equal volume of C_{60} in THF. THF was eliminated using a Buchi rotovapor (Büchi Labortechnik AG, Flawil, Switzerland) by reducing the volume to 450 mL and adding 550 mL MilliQ water. This was repeated twice, and the final solution was evaporated to 500 mL and stored overnight. The solution was filtered through a 0.22-µm nylon filter, yeilding a working nC_{60} suspension of 3.8 ppm. This suspension consisted of stable 30- to 100-nm aggregates in which the fullerenes facing the water were most likely partially modified, but the central core of the aggregate contained unmodified fullerenes (Colvin et al. 2004).

Fish exposures. Juvenile largemouth bass (*Micropterus salmoides*) were cultured at

Southern Methodist University from an initial group purchased from Tyler Fish Farms (Tyler, TX) in October 2003. These fish were covered under Animal Care and Use protocol 80207 approved by Southern Methodist University's Committee on Use of Animal Subjects. Fish were kept at 24 ± 1°C and a 14-hr:10-hr light:dark cycle, and fed Silver Cup Trout Chow #9 (Nelson & Sons, Inc., Murray, UT) *ad libitum* twice daily.

Two separate trials were performed using the same cohort of juvenile bass and the same batch of nC_{60}. A total of 28 fish were randomly assigned to either exposure or control tanks over the course of the two trials (Table 1). Fish were exposed in groups of three or four in 10-L aquaria. The smaller trial consisted of fish in one aquarium each of control [reconstituted hard water (RHW), U.S. Environmental Protection Agency (EPA) protocol (U.S. EPA 1978), containing 192 mg/L sodium bicarbonate, 120 mg/L gypsum ($CaSO_4$–$2H_2O$), 120 mg/L magnesium sulfate, and 8 mg/L potassium chloride, pH 8.5] and 0.5 ppm nC_{60} in RHW. The larger trial consisted of fish in two aquaria each of control, 0.5 ppm nC_{60}, and 100 μM hydrogen peroxide (positive control for oxidative damage) and fish in one aquarium of 1 ppm nC_{60}. Because of the high cost of production and limited quantities of nC_{60}, I exposed fish in groups of either three larger fish (control and 0.5 ppm nC_{60}) or four smaller fish (1 ppm nC_{60}) per 10-L aquarium containing 7 L exposure water. To avoid aggressive interactions, I used smaller fish in the 1-ppm exposure aquaria to allow more space for individual fish. For aquaria with three fish, the fish were physically separated from one another with glass dividers. Vigorous aeration assured that water flowed freely between all three compartments.

Water quality. A 30% water volume change and redosing was performed after 24 hr. At that point, it was apparent that the water was visibly more clear in the aquaria dosed with nC_{60} and H_2O_2 than in the control aquaria. Normally, some minor turbidity is seen in control water due to the presence of beneficial bacteria, and a Secchi reading of 80 cm is considered borderline between clear and turbid. Water clarity was measured on the removed water at 24 hr using a Secchi tube

Table 1. Summary of exposure groups and numbers and body weights of fish used in this study (both trials).

	Total no. of fish	Mean body weight ± SD (g)
Control	9	4.8 ± 2.0
0.5 ppm nC_{60}	9	5.3 ± 2.0
1 ppm nC_{60}[a]	4	2.0 ± 0.7
100 μM H_2O_2	6	5.6 ± 2.0

[a]Aquarium contained four smaller fish, compared to three fish in each of the other exposure aquaria.

(Ward's Natural Science, Rochester, NY). As an additional indicator of water quality, pH was measured at the beginning of the exposure, at the 24 hr water change, and again at the end of the exposure. The pH ranged from 8.50 to 8.56, with an average of 8.53. There was no difference in pH between the control, H_2O_2-, or nC_{60}-dosed water.

Lipid peroxidation, protein oxidation, and total GSH. After 48 hr, fish were euthanized using MS-222 (ethyl 1-aminobenzoate; Sigma Chemical Co., St. Louis, MO), and tissues were stored at –80°C until analysis. Lipid peroxidation was measured in brain, gill, and liver using the thiobarbituric acid (TBA) assay for malondialdehyde. Protein oxidation was measured in brain, gill, and liver by detecting carbonyl moieties using 2,4-dinitrophenyl-hydrazine (DNPH) and normalized to total protein. Total GSH was measured using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)–oxidized GSH (GSSG) recycling assay (Anderson 1985). Tissues were also prepared using Anderson's method. Briefly, tissue was homogenized in 4 vol 50 mM HEPES, pH 7.2, plus 0.1 mM phenylmethylsulfonyl fluoride using a Tissue Tearor (Dremel, Racine, WI) for 30 sec at full speed on ice or, if sample volumes were too small (e.g., brain tissues), by hand on ice using a glass tissue homogenizer. The homogenate was centrifuged at 14,000 × *g* for 5 min at 4°C.

For protein oxidation, nucleic acids were removed by adding 10 μL 20% streptomycin to 100 μL supernatant; after incubating at room temperature for 15 min, the DNA was removed by centrifugation at 14,000 × *g* for 5 min. Four volumes of 10 mM DNPH in 2M HCl was added to 100 μL of the DNA-free supernatant, which was incubated for 1 hr at room temperature with vortexing every 10–15 min. Proteins were precipitated and pelleted by adding 500 μL 20% trichloroactic acid and centrifuging at 14,000 × *g* for 5 min. The pellet was washed at least three times with 50:50 ethanol:ethylacetate mixture to remove any unreacted DNPH. The pellet was redissolved at 37°C in 450 μL guanidine HCl/dithiothreitol, and 200-μL aliquots were read in duplicate in a plate-reading spectrophotometer obtaining the maximum absorbance between 360 and 390 nm. The carbonyl content was calculated using a molar absorption coefficient of 22,000/M-cm = ε.

For lipid peroxidation, 1.4 mL of 0.02% butylated hydroxytoluene and 0.375% TBA were incubated with 100 μL tissue homogenates for 15 min at 100°C. The samples were cooled and spun down in a clinical centrifuge. The appearance of malondialdehyde was measured at 532 nm in 200-μL aliquots on a plate-reading spectrophotometer and was compared to a standard curve.

For total GSH, GSH was oxidized by DTNB to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). I mixed 10 μL homogenate from the peroxidation assay with 100 μL 5% sulfosalicylic acid (SSA) to denature proteases. SSA homogenate (25 μL) was incubated with 100 μL of 280 μM NADPH and 15 μL 10 mM DTNB for 10 min at 30°C to oxidize all GSH to GSSG. GSSG was then reduced by adding 15 μL GSH reductase, and the rate of TNB formation was followed at 412 nm and was proportional to the sum of GSH and GSSG present. The rate was compared with a standard curve of GSH in buffer.

Total protein was measured in tissue homogenates using the Bradford method (Bradford 1976) adapted to a 96-well plate format. Lipid peroxidation, protein oxidation, and total GSH were normalized to milligrams of protein.

Fullerenes absorb light in the ultraviolet and low visible range. To determine whether interference of fullerene absorption could account for the changes in the assays described above, I ran an absorbance spectrum of the fullerenes versus MilliQ water on a Molecular Devices Spectramax 190 plate reading spectrophotometer (Molecular Devices, Sunnyvale, CA) using a quartz microtiter plate. The spectrum was run from 190 to 850 nm.

Statistical analysis. Because three fish were exposed in each aquarium to the same dosing water, data from the three fish were pooled into aquarium averages; thus, *n* = 3 aquaria for control and 0.5 ppm nC_{60}. Data were analyzed using a two-sample *t*-test comparing aquarium averages for control and 0.5 ppm nC_{60}-exposed fish. Because there was only one aquarium for 1 ppm nC_{60} exposure and two aquaria for H_2O_2 exposures, statistics were not performed on these groups. Data were also analyzed by individual fish: *n* = 9 for both control and 0.5 ppm nC_{60} (i.e., three aquaria of three fish each), *n* = 6 for H_2O_2 (i.e., two aquaria of three fish each), and *n* = 4 for 1 ppm nC_{60} (i.e., one aquaria of four fish). Because data were not normally distributed, they were log transformed. Statistical significance was set at *p* < 0.05.

Results

Water quality. The water in the nC_{60}- and H_2O_2-dosed aquaria was more clear than the 1.2-m limit on the Secchi tube, whereas control tanks had an average Secchi depth reading of 78 cm. nC_{60} appears to be able to improve water clarity, possibly by interfering with beneficial bacterial growth. This effect of manufactured nanomaterials on microbial communities requires further study.

Lipid peroxidation, protein oxidation, and total GSH. There was a trend for reduced lipid peroxidation in liver and gill, which was

statistically significant only in analysis of individual fish as opposed to aquarium averages (Figure 1). Fish in the 1.0 ppm nC_{60}-exposure tank were smaller than the other fish (although they all came from the original cohort), which may be why there was no clear dose response. Lipid peroxidation was significantly elevated in the brain of 0.5 ppm nC_{60}-exposed fish compared with control (Figure 1). This follows well with the partitioning of nC_{60} into lipid-rich environments. There was no increase in protein oxidation in these same brain tissues or in gill or liver (Figure 2). This indicates a selective pathway of oxidative damage to lipids, and although membrane-associated proteins may also be targets for oxidative damage of nC_{60}, the cytosolic proteins may not be affected. When measuring total protein oxidation, any damage to membrane-associated proteins would then be underestimated. I found a trend for GSH depletion in gill (Figure 3), which was statistically significant only in the analysis of individual fish as opposed to aquarium averages.

Because fullerenes absorb light in the visible spectrum near the wavelengths of the above-mentioned assays, an absorbance scan of nC_{60} was run from 190 to 850 nm (Figure 4). Only the spectrum to 550 nm is shown because the fullerene absorption spectrum overlaps with the MilliQ absorption spectrum after approximately this wavelength. At the wavelength of the lipid peroxidation assay, the fullerene absorbance nearly overlays the MilliQ water absorbance, with a difference in optical density (OD) of 0.002 OD units. For comparison, absorbances in the lipid peroxidation assay range from 0.277 to 0.042 OD units. Even if fullerenes are concentrated, they would be diluted again in the actual assay, and it is highly unlikely that there would be a significant interference of the fullerenes with the assay at this wavelength. For the protein oxidation assay, the maximal difference between fullerenes and MilliQ water is 0.012 OD units. For comparison, absorbances in the protein oxidation assay range from 0.244 to 0.016 OD units. It is therefore possible that there may be minimal interference of fullerenes with this assay, even though the samples are diluted in the actual assay. If fullerenes were interfering with the assay, I would expect to find higher OD units, and therefore higher protein oxidation levels in the exposed groups, but this was not the case. On the contrary, protein oxidation levels were lower in fullerene-exposed fish than in control fish. For the GSH assay, it is the change in absorbance (rate) that is measured; therefore, even if there was additional absorption due to fullerenes (and so a higher starting OD), it would not affect the rate. Finally, in the protein assay at 595 nm, there is no difference between the absorbance of fullerenes and MilliQ water.

The H_2O_2-exposed fish showed significant ($p = 0.026$) protein oxidation of the gills (if analyzed as individual fish), as was expected of a positive control (data not shown). There were no significant protein oxidation of the liver or brain; no significant lipid peroxidation of brain, liver, or gill; and no significant differences in GSH levels in the gill or liver of H_2O_2-exposed fish compared with the controls. This shows that H_2O_2 is a good positive control for oxidation of the gill, which is in direct contact with the H_2O_2, but not of liver or brain, which are not in direct contact with the H_2O_2.

Discussion

Although I found no changes in total protein oxidation in any of the tissues, there were changes in lipid peroxidation. In gill and liver, I observed a trend for decreased lipid peroxidation, possibly due to induction of repair enzymes. Further studies on gene expression changes are currently under way to investigate this possibility. In the brain, I found a significant increase in lipid peroxidation in 0.5 ppm nC_{60}–exposed fish compared with controls. The differences between brain, gill, and liver are striking. The gill and liver showed a trend toward decreased lipid peroxidation, whereas
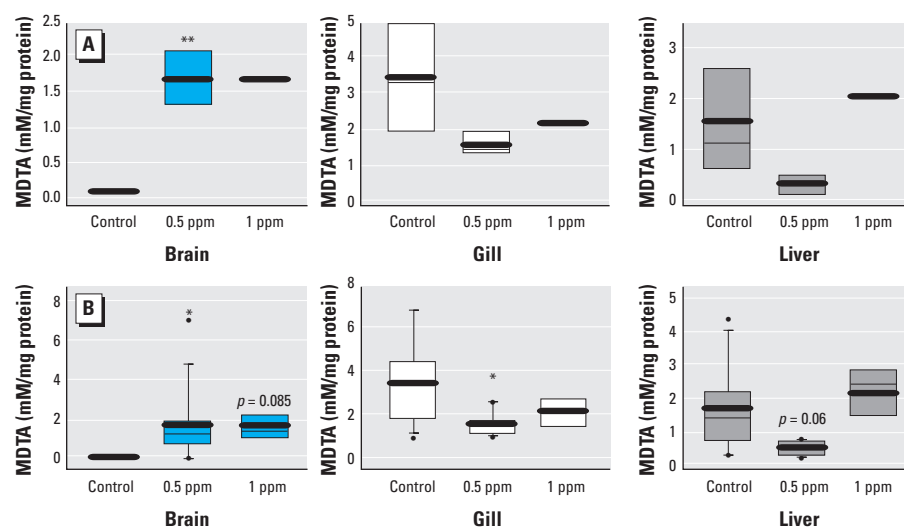

Figure 1. Lipid peroxidation of brain, gill, and liver of largemouth bass after 48 hr of exposure to 0.5 or 1 ppm nC_{60}. MDTA, 1,1,3,3-tetraethoxy propane. (*A*) Aquarium averages. (*B*) Data using individual fish; in the brain, 0.5 ppm nC_{60} caused a significant 17-fold increase in lipid peroxidation, whereas in gill and liver there is a trend for reduction of lipid peroxidation at 0.5 ppm nC_{60}. Heavy black bands represent the means; thinner lines indicate medians; boxes represent 25th and 75th percentiles; error bars indicate minimum and maximum; and circles represent outliers.
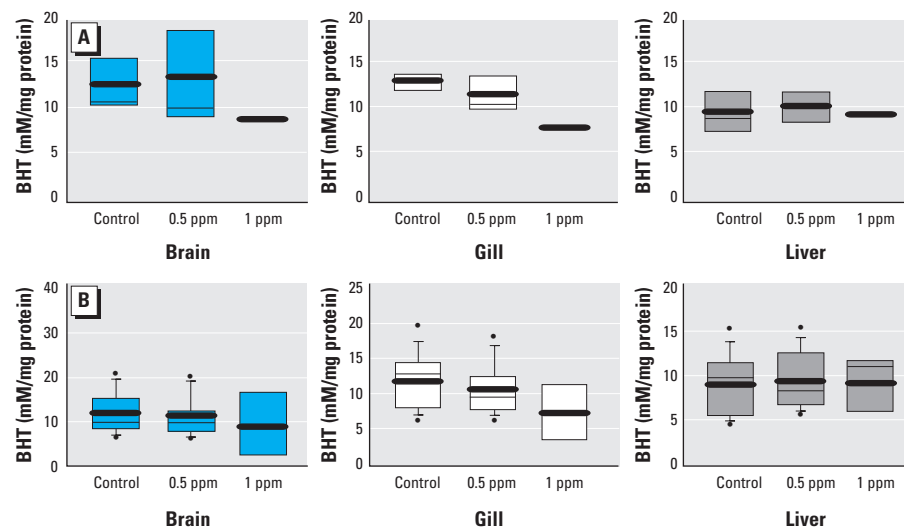*$p < 0.05$. **$p < 0.01$.

Figure 2. Protein oxidation of brain, gill, and liver of largemouth bass after 48 hr exposure to 0.5 or 1 ppm nC_{60}. BHT, butylated hydroxytoluene. (*A*) Aquarium averages. (*B*) Data using individual fish. Heavy black bands represent the means; thinner lines indicate medians; boxes represent 25th and 75th percentiles; error bars indicate minimum and maximum; and circles represent outliers.

the brain had significantly elevated lipid peroxidation. Other nano-sized particles can be selectively transported to the brain via the olfactory neuron in mammals (Bodian and Howe 1941; DeLorenzo 1970; Howe and Bodian 1941; Oberdörster et al. 2004). Indeed, as described by Tjälve et al. (1995), soluble $^{54}Mn^{2+}$ dosed into the olfactory chamber of pike was taken up into the olfactory receptor cells and was transported at a maximal rate of 2.90 ± 0.21 mm/hr into the olfactory bulb. It is possible that fullerenes are also mobilized in this manner to the brain. One possible reason that gills and liver of the nC_{60}-exposed fish did not show increased lipid peroxidation may be due to better antioxidant

defenses in these organs. In addition, it is conceivable that colloidal fullerenes need to be transported to lipid-rich regions (e.g., brain) before the colloid dissociates and frees individual redox-active fullerenes. It is also possible that there may be an inflammatory response creating reactive oxygen species (ROSs) or that a reactive fullerene metabolite is produced. The actual mechanism still needs to be determined, and future research will focus on this question.

The ability of fullerenes to selectively localize to membranes makes them an interesting option as a drug-delivery device (Foley et al. 2002); at the same time, however, because fullerenes are also redox active, one would expect damage in lipid-rich tissues. The balance between the desirable therapeutic and potential toxic effects may be determined by the persistence of biocompatible coatings. Because fullerenes in the present study were uncoated, the full potential of redox-active surfaces was present. To date, studies of biologic associations with fullerenes have only been done *in vitro*, and although this is a logical and an important first step, the whole-body dispersion of fullerenes will become an issue once they are released into the environment or if they are administered directly for therapeutic purposes. Further *in vivo* studies with fullerenes and other manufactured nanomaterials will have to be carried out to determine whole-body tissue distributions, and potential corresponding effects, to determine their safety.

In the fish, the trend for GSH depletion in gill parallels what has been shown by others after *in vitro* exposure of nano-sized ultrafine particulate matter to both murine macrophages and transformed human bronchial epithelial cells (Li et al. 2003). The depletion of GSH is used as an indication of oxyradical scavenging ability, showing that the antioxidant defense system is overwhelmed by ROSs. The liver has a much higher capacity to deal with ROSs, as is indicated by the higher levels of GSH in control liver (> 100 μM GSH/mg protein) compared with control gill (~ 3 μM GSH/mg protein). This trend for depletion of total GSH may be an indication that the antioxidant defense system of the gill is being stressed.

The improvement of water clarity with both 0.5 and 1 ppm nC_{60} suggests that these uncoated fullerenes may be bactericidal to beneficial bacteria normally found in aquaria. To date, no published studies have reported on the bactericidal properties of fullerenes, and this would be an important area of research, especially as it relates to microbial community changes.

This is the first study showing that manufactured nanomaterials can have adverse effects on aquatic organisms, and it is possible that effects in fish may also predict potential effects in humans. Given the rapid onset of brain lipid peroxidation, it is important from a preventative
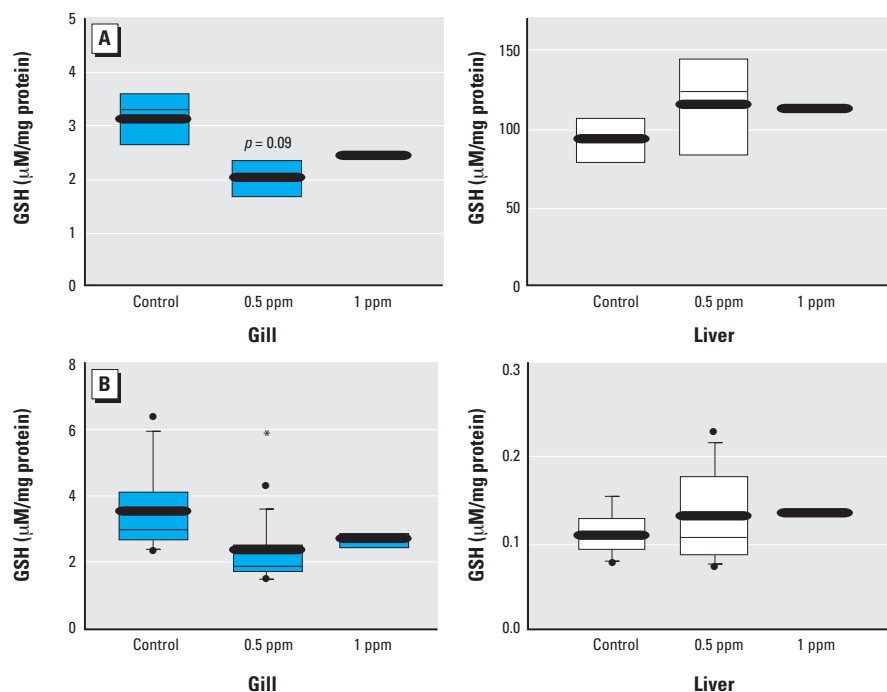

Figure 3. Total GSH in gill and liver of largemouth bass after 48 hr exposure to 0.5 or 1 ppm nC_{60}. (*A*) Aquarium averages. (*B*) Data using individual fish. GSH shows a trend for depletion in gill tissues, based on analysis of individual fish as opposed to aquarium averages. Heavy black bands represent the means; thinner lines indicate medians; boxes represent 25th and 75th percentiles; error bars indicate minimum and maximum; and circles represent outliers.
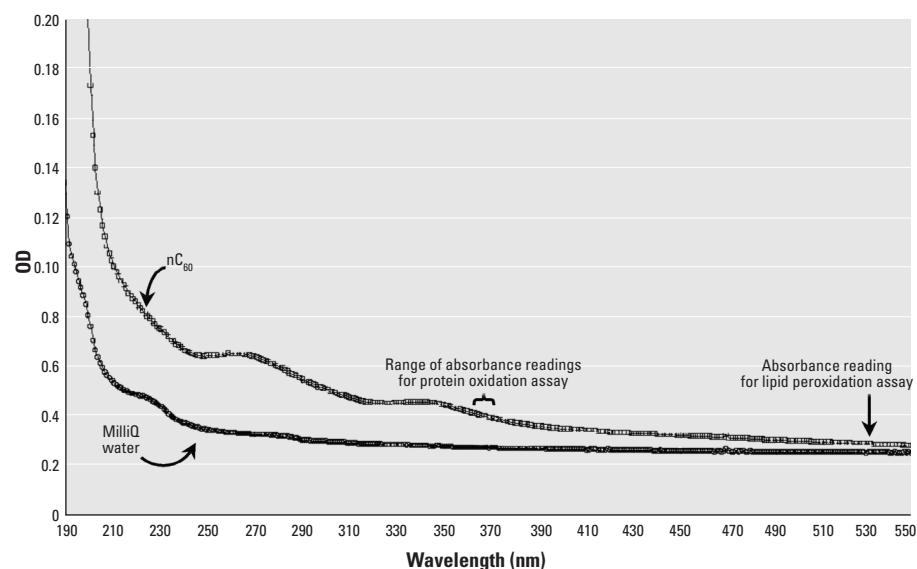*$p < 0.05$.

Figure 4. Absorbance of nC_{60} compared with MilliQ water, showing the typical fullerene absorption spectrum. OD, optical density. At the wavelengths used for the protein oxidation assay (364–370 nm), the nC_{60} has a slightly higher absorbance reading (maximum of 0.012 OD units), whereas at the lipid peroxidation wavelength (532 nm), the nC_{60} has a *de minimis* difference in absorbance reading (0.002 OD units).

point of view to further test manufactured nanomaterials before they are used by humans and in industrial applications. If such preventative principles had been applied to compounds such as DDT and polychlorinated biphenyls, significant environmental damage could have been avoided. The present study is only the first step in assessing the potential biologic impacts on aquatic species by manufactured nanomaterials, and the effect of manufactured nanomaterials on microbial communities should be further investigated. Studies on the fate and effects of nanomaterials in the environment and in living organisms are needed to more clearly define the benefits and potential risks of this promising technology.

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