

*Nanomaterials in the Environment*TOXICITY OF AQUEOUS C<sub>70</sub>-GALLIC ACID SUSPENSION IN *DAPHNIA MAGNA*BRANDON C. SEDA,<sup>\*†</sup> PU-CHUN KE,<sup>†‡</sup> ANDREW S. MOUNT,<sup>†§</sup> and STEPHEN J. KLAINET<sup>†§</sup><sup>†</sup>Institute of Environmental Toxicology, Clemson University, Pendleton, South Carolina, USA<sup>‡</sup>Department of Physics and Astronomy, Clemson University, Clemson, South Carolina, USA<sup>§</sup>Department of Biological Sciences, Clemson University, Clemson, South Carolina, USA

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**Abstract**—The present study assessed the toxic effects of stable aqueous colloidal suspensions of gallic-acid-stabilized C<sub>70</sub> fullerene on *Daphnia magna*. The suspensions were stabilized through noncovalent surface modification with gallic acid. In addition to whole-organism responses, changes in antioxidative processes in *D. magna* were quantified. Acute toxicity was observed with 96LC50 for C<sub>70</sub>-gallic acid of 0.4 ± 0.1 mg/L C<sub>70</sub>. *Daphnia magna* fecundity was significantly reduced in 21-d bioassays at C<sub>70</sub>-gallic acid concentrations below quantifiable limits. Antioxidant enzyme activities of glutathione peroxidase and superoxide dismutase as well as lipid peroxidation suggested that exposed organisms experienced oxidative stress. Microscopic techniques used to determine cellular toxicity via apoptosis proved unsuccessful. Environ. Toxicol. Chem. 2012;31:215–220. © 2011 SETAC

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## INTRODUCTION

The burgeoning development of nanotechnology allows for a wide range of applications, with potentially exponential growth in production and use, leading to considerable discharge of nanomaterials into the environment. Understanding how these materials interact in the environment is of particular importance in determining bioavailability to organisms. Carbon nanomaterials discharged into aquatic systems can become functionalized or derivatized by biomolecules and natural organic matter (NOM) [1]. Salonen et al. [2] showed that gallic acid (GA), a ubiquitously occurring component of NOM, self-assembled with the fullerene C<sub>70</sub> to form stable aqueous suspensions. Furthermore, these authors demonstrated translocation of these surface-modified fullerenes into mammalian cells and observed consequential contraction of the cell membranes. These observations laid the foundation for the present research.

Much of the existing research on the ecotoxicology of fullerenes has focused on the effects of C<sub>60</sub> and derivatives of C<sub>60</sub> [3]. The effects of the fullerene C<sub>70</sub> are not as well characterized. Fullerenes, such as C<sub>70</sub>, may have toxicological effects similar to those of C<sub>60</sub> because they share similar closely related physical and chemical properties.

Conflicting opinions exist about the toxicity of C<sub>60</sub> in aquatic organisms. Suspended C<sub>60</sub> has been observed to induce oxidative damage in human cells and in aquatic organisms [4,5]. Other research has indicated, however, that, depending on surface modification and method of preparation, these C<sub>60</sub> can actually act as highly effective antioxidants by radical scavenging [6–8]. Foley et al. [9] reported that although fullerenes can quench reactive oxygen species (ROS), they will

produce singlet oxygen when illuminated with ultraviolet (UV) radiation. Therefore, oxidative stress might be a significant contributing factor to C<sub>70</sub> toxicity, but might not be the exclusive cause.

The ability of different colloidal C<sub>60</sub> suspensions to produce ROS depends on the solvent used for their preparation and environmental conditions (that is, UV radiation) [5,10]. However, evidence also shows that C<sub>60</sub>, depending on derivatization and method of suspension, can act as an antioxidant [11–13]. In cells exposed to C<sub>60</sub>, cell death occurs because of lipid oxidation caused by generating oxygen radicals; highly derivatized C<sub>60</sub> systems do not generate these species as readily and thus have lower cellular toxicity [5]. Both lipid-soluble and water-soluble C<sub>60</sub> derivatives effectively prevent lipids from radical-initiated peroxidation and the breakdown of membrane integrity [13].

To minimize oxidative damage to cellular components resulting from ROS production, organisms have developed antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) [14,15]. Upregulation of these antioxidant enzymes within organisms can occur in response to elevated ROS production: differences in how these enzymes are regulated can be observed as biomarkers of ROS levels [16]. Failure to moderate excessive ROS can lead to deleterious effects such as enzyme inactivation, protein degradation, DNA damage, and lipid peroxidation [15]. Lipid peroxidation is of particular concern as a cause of tissue damage, which can lead to disrupting essential cellular functions [17]. Malondialdehyde, a byproduct of lipid peroxidation, can be measured as an oxidative stress biomarker [18]. *Daphnia magna*, a widely used test organism for aquatic risk assessment, has been shown to express such biomarkers as changes in enzyme regulation and lipid peroxidation resulting from exposure to UV light, redox cycling compounds, and transition metals [19,20].

These antioxidative measures require energy, which would otherwise be used for physiological functions such as reproduction. These functions may also be impaired by carbon nanomaterials via physical stress. For instance, carbon nanomaterials such as carbon nanotubes can accumulate in the gut lumen, possibly reducing efficient absorption of food [21,22].

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Therefore, in addition to oxidative damage, C<sub>70</sub> potentially causes other sublethal effects on organism fitness.

The goal of the present study was to assess the response of *D. magna* to exposures of GA-modified C<sub>70</sub> fullerene. To accomplish this, we established and met the following objectives: characterize the response of *D. magna* to acute and chronic exposures of C<sub>70</sub>-gallic acid (C<sub>70</sub>-GA) and quantify the oxidative damage following sublethal C<sub>70</sub>-GA exposure.

## MATERIALS AND METHODS

### *Suspension of C<sub>70</sub>-gallic acid*

Fullerene C<sub>70</sub> was purchased from SES Research, and its purity was reported by the distributor to be 95%. To prepare suspensions of C<sub>70</sub>-GA, 5 mg C<sub>70</sub> and 25 mg GA were combined in a 100-ml conical glass tube. Increments of synthetic laboratory moderately hard freshwater (MHW) were added to obtain a final volume of 100 ml [23]. The contents of the tube were probe sonicated for 1 h. Suspensions were allowed to settle for 1 h, and the supernatant was transferred to a clean glass tube using a glass pipet. Care was taken not to transfer settled particles. All exposure suspensions were prepared assuming a nominal stock concentration of C<sub>70</sub>-GA (50 mg/L C<sub>70</sub>). Because the suspension procedure was not 100% effective, exposure concentrations were quantified as described below.

### *Characterization of particle size*

Test suspensions of C<sub>70</sub>-GA complex (nominal concentration 10 mg/L C<sub>70</sub>) were analyzed for particle size using dynamic light scattering. A Beckman Coulter Photon Correlation Spectroscopy submicrometer particle size analyzer was used for dynamic light scattering analysis. A Zeta Sizer Z (Malvern) was used to measure zeta potential of C<sub>70</sub> and C<sub>70</sub>-GA suspensions. For each characterization, care was taken to avoid samples containing unsuspended materials.

### *Quantification of C<sub>70</sub>-GA test suspensions*

Samples of test suspensions were obtained prior to each daily medium renewal to determine the C<sub>70</sub> concentration. Twenty-five-milliliter samples were shaken vigorously with 5 ml hexane to extract the C<sub>70</sub>. A single aliquot was obtained from each daily-prepared treatment suspension concentration. Extracts were stored in 7-ml glass vials. Concentrations of C<sub>70</sub> in hexane extracts were quantified spectrophotometrically ( $\lambda = 550$  nm) using a Molecular Devices SpectraMAX Gemini UV spectrophotometer.

A standard curve was prepared (Supplemental Data) using stock C<sub>70</sub>-GA dissolved in hexane. C<sub>70</sub>-GA concentrations were calculated as milligrams C<sub>70</sub> detected per liter aqueous test suspension. The lowest concentration used for the standard curve was 0.015 mg/L C<sub>70</sub> in hexane, so calculated suspension concentrations less than 0.03 mg/L C<sub>70</sub> were considered beneath the detectable limit of quantification. Concentrations were averaged over the duration of each experiment.

### *Daphnid acute bioassays*

*Daphnia magna* neonates were obtained from an in-house laboratory stock maintained at the Institute of Environmental Toxicology, Clemson University (CU-ENTOX). Routine reference acute toxicity tests have been performed previously with this culture to ensure consistent culture sensitivity to sodium chloride. Results of these reference toxicity tests are available through CU-ENTOX by contacting the corresponding author.

Tests were performed based on U.S. Environmental Protection Agency standard methods using synthetic freshwater [23]. Test volumes and the number of organisms per replicate were altered to compensate for the limited supply of C<sub>70</sub>. This synthetic freshwater was used to prepare C<sub>70</sub>-GA suspensions of the following nominal test concentrations: 10, 8, 4, 2, and 1 mg/L C<sub>70</sub> by serial dilution for acute toxicity bioassays. Test suspensions were prepared daily.

Acute bioassay methods followed standard procedures [23]. *Daphnia magna* neonates aged less than 24 h were exposed in static renewal acute toxicity tests in 30-ml glass beakers containing 25-ml test solutions at 25 ± 1°C. Three replicates per treatment were tested, and each treatment included five neonates. Mortality was observed at 24-h intervals with organisms fed a diet of algae (*Selenastrum capricornutum*) and yeast-cereal-trout chow. After allowing organisms to feed for 1 h, all living organisms were transferred to test chambers containing fresh test solutions.

### *Daphnid chronic bioassays*

Chronic bioassay methods followed standard procedures [23,24], with modifications. Test suspensions of C<sub>70</sub>-GA of nominal test concentrations 2, 1, 0.5, 0.25, and 0.125 mg/L C<sub>70</sub> were prepared by serial dilution of stock suspension with synthetic freshwater for the 21-d bioassays. Neonates aged less than 24 h were exposed in static renewal tests in 500-ml polyethylene beakers containing 400-ml test solutions at 25 ± 1°C. Three replicates of five individuals were tested per treatment. Mortality and reproduction were observed at 24-h intervals. At this time, all offspring were counted and discarded, and remaining living organisms were transferred to test chambers containing fresh test solutions. After daily transfer, organisms were fed a diet of algae (*S. capricornutum*) and yeast-cereal-trout chow.

### *Lipid peroxidation*

For each treatment, 20 *D. magna* neonates (24 h old) were placed in each of three 500-ml polyethylene beakers containing 400 ml of appropriate test solutions: C<sub>70</sub>-GA (0.5 and 2.5 mg C<sub>70</sub>), 10 mg/L GA, and control. Synthetic MHW and a solution of 50 mg/L GA were each used as controls. Each treatment was carried out in three replicates. After static exposure for 24 h, organisms from each experiment were subsampled for analysis of lipid peroxidation. Lipids were extracted from whole-body daphnids (pooled wet mass 100–200 mg,  $n = 3$ ), with the extent of lipid peroxidation determined by the thiobarbituric acid-reactive species (TBARS) assay according to the procedure of Barata et al. [20]. Measurements were carried out with a fluorescence spectrometer.

### *Enzyme activity and protein determination*

Juvenile *D. magna* (4–5 d old) were exposed for 24 h as in the test described in the Lipid peroxidation section above. Treatments included 10 mg/L GA solution, a C<sub>70</sub>-GA suspension (nominal concentration of 2 mg/L C<sub>70</sub>), and 20 µg/L Cu solution. Copper was used as a positive control as a known oxidative stressor that results in changes in TBARS and antioxidant enzyme expressions [20]. Three replicate tests were performed for each treatment. Gallic acid and copper solutions were prepared using MHW. Juveniles (pooled wet mass 50–100 mg) from each sample were homogenized in 1:4 wet weight:buffer volume ratio in 4°C 100 mM KCl, pH 7.4, and 1 mM ethylenediaminetetraacetic acid.

Homogenates were centrifuged at 10,000 g for 10 min and the supernatants removed for immediate enzyme activity analysis. Measurements were carried out on a plate reader spectrophotometer (Molecular Devices SpectraMax 190) at 25°C. Superoxide dismutase activity was measured by SOD assay kit (Cayman Chemical) according to kit instructions. Glutathione peroxidase activity was determined using a GPX assay kit (Cayman Chemical) according to kit instructions. Protein concentrations in the supernatants were measured using a modified Lowry Protein Assay kit (Pierce).

#### Apoptosis

Juvenile *D. magna* were exposed to C<sub>70</sub>-GA (nominal concentration of 2 mg/L C<sub>70</sub>) for 24 h. For each treatment and control, 10 individuals were fixed in 10% buffered paraformaldehyde. Samples were embedded in paraffin, and then cross-sections were prepared by ultramicrotome. Samples were prepared and analyzed according to the manufacturer's instructions for the ApopTag ISOL Dual Fluorescence Apoptosis Detection kit (Chemicon). Analysis was performed on a Zeiss 510 laser scanning confocal fluorescent microscope.

#### Statistical analysis

Survival data from the 96-h test were analyzed using the trimmed Spearman–Karber method to derive 96-h median lethal concentration (LC50). Data sets from the 21-d toxicity bioassays, lipid peroxidation, and enzyme activity levels were each analyzed for significance by one-way ANOVA with Tukey's post hoc test in SAS Software (SAS Institute). Apoptosis data were analyzed for significance using Student's *t* test. Significant differences were established at *p* < 0.05.

## RESULTS AND DISCUSSION

#### C<sub>70</sub>-GA suspension characterization

We produced colloidal suspensions of C<sub>70</sub>-GA in MHW to use in the *D. magna* toxicity bioassays by modifying the method of Salonen et al. [2] for coating C<sub>70</sub> with GA. While quantification methods did not account for 100% of the C<sub>70</sub> placed in suspension, we were able to quantify the average C<sub>70</sub> concentrations in treatments by hexane extraction. Measured concentrations were approximately 10% of nominal concentrations, indicating that a considerable amount of C<sub>70</sub> did not remain suspended after sonication of the stock suspensions. The mean particle size of a C<sub>70</sub>-GA test solution 2 h after preparation was calculated as 1,432 ± 690 nm (Fig. 1). The size distribution corresponds to the tens of nanometers up to micrometers range

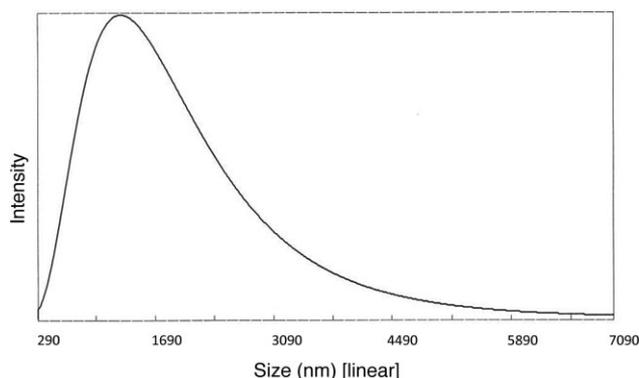


Fig. 1. Particle size distribution of C<sub>70</sub>-gallic acid determined by dynamic light scattering within 2 h of preparation. Mean particle size of 1,432 ± 690 nm.

of particle sizes reported by Salonen et al. [2] for unfiltered C<sub>70</sub>-GA in deionized water.

Zeta potentials for C<sub>70</sub> and C<sub>70</sub>-GA suspended in MHW were  $-29 \pm 7.8$  mV and  $-32 \pm 7.2$  mV, respectively. These values are not significantly different and indicate that both methods produced suspensions considered incipiently to moderately stable. However, a visible portion of C<sub>70</sub> had precipitated from the suspension without GA prior to zeta potential analysis.

On visual inspection of suspensions prepared in MHW compared with those prepared as described by Salonen et al. [2] with deionized water, larger aggregates were observed in suspensions prepared in MHW. A possible explanation for the increase in the suspensions in the present study is the greater ionic strength of MHW compared with distilled water. Ionic strength has been shown to increase aggregation of fullerene suspensions in water [25].

#### Acute toxicity of C<sub>70</sub>-GA

A dose-dependent decrease in survival was observed for *D. magna* neonates with a 96-h LC50 value of  $0.4 \pm 0.1$  mg/L (Fig. 2). Microscopic examination of the gut tract of exposed organisms indicated substantial amounts of nanoparticles, which coincides with previous data showing that *D. magna* collected nanomaterial aggregates within their gut tract [21,22]. We could not confirm, however, that C<sub>70</sub>-GA migrated beyond the epithelia of the gut tract.

Time-course micrographs (Supplemental Data) indicated rapid accumulation of fullerenes within the gut tract, followed by clearance after the individual was placed in clean MHW. Depuration times of greater than 12 h were required for the material to clear completely from the gut tract. This time is greater than that reported for clearance of suspended clay by *D. magna* [26], but shorter than the 28 h reported for multi-walled carbon nanotubes suspended in NOM [22]. However, given that C<sub>70</sub>-GA in the present study was more toxic than the multiwalled carbon nanotubes reported by Edgington et al. [22], it is likely that something beyond gut tract clogging and interference with food processing is causing the toxicity.

#### Chronic toxicity of C<sub>70</sub>-GA

*Daphnia magna* exposed to C<sub>70</sub>-GA for 21 d exhibited significant mortality at fullerene concentrations  $\geq 1$  mg/L

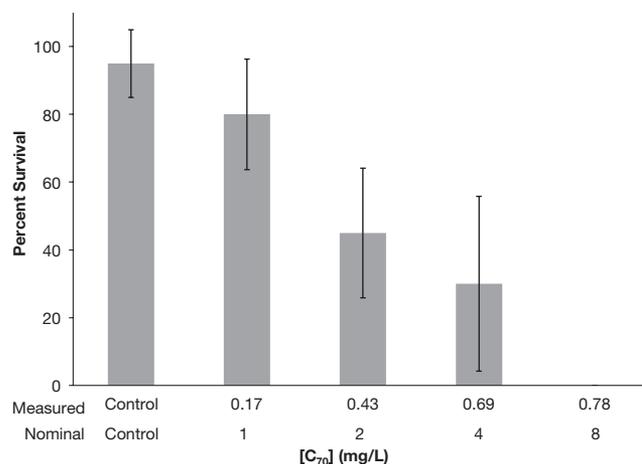


Fig. 2. Mean percentage survival ( $\pm$ SD) of *Daphnia magna* exposed to C<sub>70</sub>-gallic acid complex (C<sub>70</sub>-GA) in a 96-h static renewal test. The x axis concentrations represent average [C<sub>70</sub>] of suspensions calculated from milligrams C<sub>70</sub> extracted in hexane per liter of aqueous suspension.

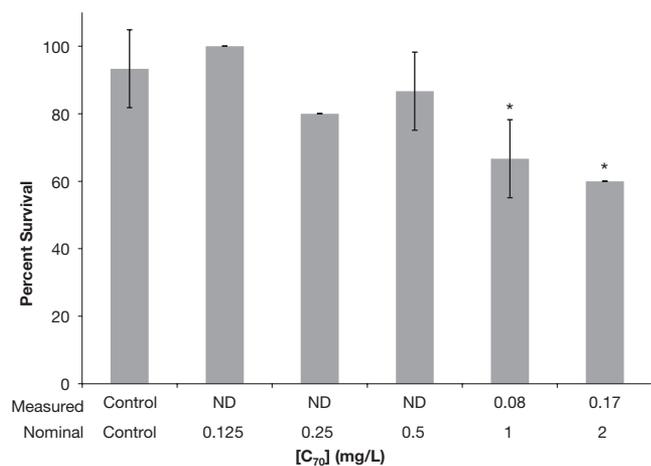


Fig. 3. Mean percentage survival ( $\pm$ SD) of *Daphnia magna* exposed to  $C_{70}$ -gallic acid complex ( $C_{70}$ -GA) in a 21-d static renewal bioassay. The  $x$  axis concentrations represent average  $[C_{70}]$  of suspensions calculated from milligrams  $C_{70}$  extracted in hexane per liter of aqueous suspension. Concentrations below the limits of the standard curve (0.03 mg/L) are labeled as ND. \*Significantly greater than control ( $p < 0.05$ ).

(Fig. 3). Sublethal effects of a 21-d  $C_{70}$ -GA exposure on *D. magna* indicate a significant decrease in fecundity at concentrations  $< 0.1$  mg/L (Fig. 4). The use of organic solvents such as tetrahydrofuran in preparing aqueous fullerene suspensions has been controversial, because this solvent has been shown to exhibit neurotoxicity [27]. However, GA is a widely used antioxidant and did not exhibit significant toxicity when tested alone in this study. The surface-modified fullerenes tested in the present study exhibited toxicity to *D. magna* similar to that of the  $C_{60}$  tested by Oberdörster et al. [4] prepared with tetrahydrofuran (5 ppm  $C_{60}$  clusters).

Few other studies have reported the effects of chronic exposure of fullerenes to aquatic organisms. For example, juvenile carp exposed for 32 d to 0.2 mg/L  $C_{60}$  exhibited reduced body length and weight [28]. To the best of our knowledge, this is the first study to report a significant reproductive effect on a eukaryotic organism resulting from fullerene exposure. Indeed, Ringwood et al. [29] postulated that fullerene

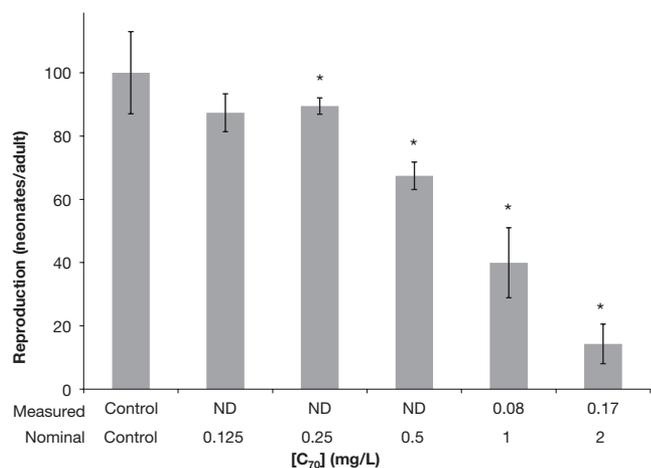


Fig. 4. Mean number of neonates per adult ( $\pm$ SD) of *Daphnia magna* exposed to  $C_{70}$ -gallic acid complex ( $C_{70}$ -GA) in a 21-d static renewal test. The  $x$  axis concentrations represent average  $[C_{70}]$  of suspensions calculated from milligrams  $C_{70}$  extracted in hexane per liter of aqueous suspension. Concentrations below the limits of the standard curve (0.03 mg/L) are labeled as ND. \*Significantly greater than control ( $p < 0.05$ ).

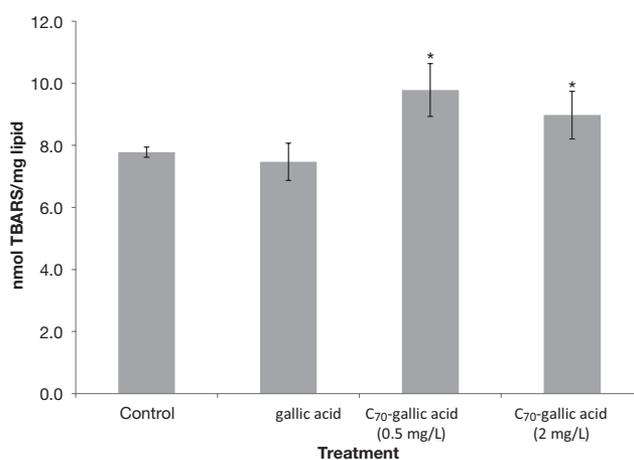


Fig. 5. Mean thiobarbituric acid-reactive species detected as relative to mass of lipid extracts ( $\pm$ SD) of *Daphnia magna* exposed to  $C_{70}$ -gallic acid ( $C_{70}$ -GA) in a 24-h static test ( $n = 3$ ). Treatments include moderately hard water control, GA, and nominal concentrations of  $C_{70}$ -GA: 0.5 and 2 mg/L  $C_{70}$ . \*Significantly greater than control ( $p < 0.05$ ).

exposures greater than 10  $\mu$ g/L could reduce the reproductive success of adult oysters.

The observed decrease in fecundity of *D. magna* exposed to  $C_{70}$ -GA could be a result of energetic effects caused by interference with food processing as previously proposed for single-walled nanotube aggregates [21]. In addition, should the observed elevated rates of enzyme activity continue over the course of the *Daphnia* life cycle, the energy required for reproduction could be reallocated to maintain these enzymatic pathways.

#### Lipid peroxidation

The presence of malondialdehyde, a product of lipid peroxidation that reacts with thiobarbituric acid, was determined for each treatment by the TBARS assay. No significant difference in lipid peroxidation was observed with the GA treatment.  $C_{70}$ -GA significantly increased lipid peroxidation, but the effect was not dose dependent (Fig. 5).

From the data, no dependence on concentration could be determined, suggesting that at least one limiting factor exists for the induction of lipid peroxidation in *D. magna* by  $C_{70}$ -GA. A contributing factor that might constrain lipid peroxidation could be that much of the  $C_{70}$ -GA is retained unabsorbed within the gut tract. If  $C_{70}$ -GA was absorbed by *D. magna*, the amount could be limited by the gut epithelium.

#### Antioxidant enzyme activity

*Daphnia magna* exposed to  $C_{70}$ -GA exhibited increased SOD and GPX activity (Figs. 6 and 7, respectively). Juveniles exposed to GA alone were observed to have a decreased SOD activity response. This decreased activity could be due to the fact that GA can act as an antioxidant. However, no significant change in GPX activity was observed in *D. magna* juveniles exposed to GA alone. Because GA was observed to have an opposite action on GPX activity, the GA may have an ameliorative effect counter to the apparent oxidative stress caused by the  $C_{70}$ -GA.

#### Apoptosis

The ApopTag ISOL dual fluorescence apoptosis detection kit (Chemicon) assay is designed to detect DNA fragmentation

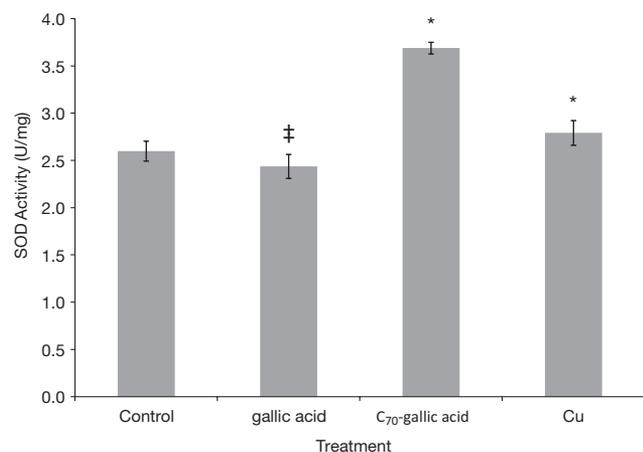


Fig. 6. Activity of superoxide dismutase (SOD) in *D. magna* juveniles exposed to gallic acid, C<sub>70</sub>-gallic acid (C<sub>70</sub>-GA), and Cu ( $n = 3$ ). Values are expressed as mean  $\pm$  SD normalized to protein concentration. ‡Significantly less than control ( $p < 0.05$ ). \*Significantly greater than control ( $p < 0.05$ ).

resulting from two DNase classes. However, autofluorescence of *D. magna* tissue samples was detected in the same fluorescence wavelength range as the DNase type II label probe carboxyfluorescein. Therefore, only Cal Fluor Red 590-labeled probes bound to fragmented DNA resulting from type I nucleases were detected.

Fluorescence micrographs of cross-sections of both control and C<sub>70</sub>-GA-exposed *D. magna* juveniles were observed for fluorescence associated with label probes. Only cells of the gut tract were considered for analysis for ease of tissue identification and proximity to C<sub>70</sub>-GA aggregates. There was no significant difference between mean apoptotic cell counts between control and C<sub>70</sub>-GA-exposed *D. magna* juveniles (Supplemental Data). From the data, no evidence emerged of a significant induction of apoptosis in *D. magna* gut tissue by the C<sub>70</sub>-GA complex.

### CONCLUSIONS

Results of this work demonstrate that chronic exposure to C<sub>70</sub>-GA can cause deleterious effects on fecundity in

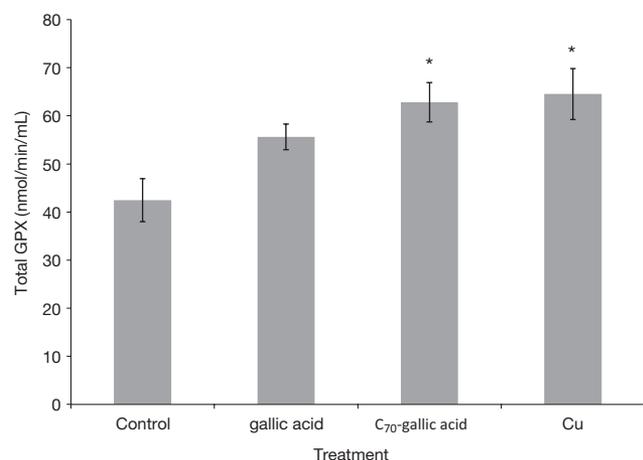


Fig. 7. Activity of glutathione peroxidase (GPX) in *D. magna* juveniles exposed to gallic acid, C<sub>70</sub>-gallic acid, and Cu ( $n = 3$ ). Values are expressed as mean  $\pm$  SD normalized to protein concentration. \*Significantly greater than control ( $p < 0.05$ ).

*D. magna* at concentrations below 0.03 mg/L C<sub>70</sub>, which was the detection limit of our quantification assay. These chronic effects may result from physical stress of fullerene aggregates in the gut, impeding the ability of individuals to feed efficiently. It is likely, however, that this physical effect is not the only adverse outcome, because C<sub>70</sub>-GA exhibited toxicity at lower concentrations despite having a shorter gut tract clearance time [22]. This mechanism could be oxidative stress, as suggested by the results of the biochemical assays in the present study. Previous studies examining fullerene-induced oxidative stress might have been confounded by the use of solvents such as tetrahydrofuran and dimethylsulfoxide to produce the aqueous suspensions [30]. Whereas these solvents were inherently toxic, the GA used in the present study to stabilize C<sub>70</sub> was not toxic. Results of the present study underscore the need for additional chronic studies with nanomaterials on aquatic organisms.

### SUPPLEMENTAL DATA

#### Supplemental Data (1.13 MB PDF).

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