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Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C₆₀, and C₆₀HxC₇₀Hx)

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Abstract

Little is known about the impact manufactured nanoparticles will have on aquatic organisms. Previously, we demonstrated that toxicity differs with nanoparticle type and preparation and observed behavioral changes upon exposure to the more lethal nanoparticle suspensions. In this experiment, we quantified these behavioral and physiological responses of *Daphnia* at sublethal nanoparticle concentrations. Titanium dioxide (TiO₂) and fullerenes (nano-C₆₀) were chosen for their potential use in technology. Other studies suggest that addition of functional groups to particles can affect their toxicity to cell cultures, but it is unknown if the same is true at the whole organism level. Therefore, a fullerene derivative, C₆₀HxC₇₀Hx, was also used to examine how functional groups affect *Daphnia* response. Using a high-speed camera, we quantified several behavior and physiological parameters including hopping frequency, feeding appendage and postabdominal curling movement, and heart rate. Nano-C₆₀ was the only suspension to cause a significant change in heart rate. Both exposure to nano-C₆₀ and C₆₀HxC₇₀Hx suspensions caused hopping frequency and appendage movement to increase. These results are associated with increased risk of predation and reproductive decline. They indicate that certain nanoparticle types may have impacts on population and food web dynamics in aquatic systems.

Keywords

Daphnia; behavior; toxicity; nanoparticle; fullerene; zooplankton; titanium dioxide

INTRODUCTION

The impact of the release of various nanoparticles into the environment is relatively unknown, but has become a prominent question in nanoparticle research(1). While the advancements in technology may be considerable, there is also concern about unintended effects of exposure to nanomaterials(1). With increased use of nanomaterials in various human products, there could be an increased possibility of their release into the environment. Aquatic environments may be particularly vulnerable due to the potential for rapid mixing and dispersal of nanomaterials entering the system as effluent from industry or personal waste water as well as rainwater runoff. Little is known about the influence these nanoparticles will have on aquatic organisms (2) or how to make predictions of their possible impacts.

There has been some indication that nanoparticle toxicity varies with particle type as well as functional groups attached to particles. Recently, Lovern and Klaper(3) observed mortality of

Daphnia magna after nanoparticle exposure differs with particle type. Fullerenes caused mortality at doses as low as 260 parts per billion (ppb) and 50% mortality at 460ppb. This study also found toxicity of nanosized TiO₂ at 2.0 parts per million and 50% mortality at 5.5 parts per million. In addition, studies have shown that toxicity of nanomaterials to cell cultures is decreased by adding functional groups to fullerenes(4) and carbon nanotubes(5). However, this has not been tested *in vivo* at the organism level.

Several toxic substances have been shown to cause changes in zooplankton behavior, thereby influencing predation risk and ultimately population dynamics(6). Additionally, behavior has been shown to be an early and sensitive indicator of toxicity at ecologically relevant concentrations(7). In our previous work, we observed *Daphnia magna* exhibiting abnormal behavior such as sporadic swimming and increased escape maneuvers upon exposure to nanoparticle suspensions(3) which could lead to an increase in mortality as changes in *Daphnia* swimming behaviors have been shown to affect predation risk(8).

In this study, we quantified these behaviors in *Daphnia magna* at levels of nanoparticles that were previously shown to be sublethal to the organism(3). Heart rate, hopping rate, feeding appendage beat frequency, and postabdominal curling were measured over several intervals. *Daphnia* heart rate has been used as an indication of physiological effects in studies on temperature(9) and chemical exposure(10). Additionally, it has been suggested that sublethal concentrations of toxicants may change individual physiological behaviors that have long-term effects at the population level(11).

Daphnia movement such as hopping rate plays a significant role in aquatic trophic relationships by affecting predation rate(12). Decreased movement of a zooplankton will diminish the ability of the predator to locate its prey(13), thereby decreasing predation risk, while irregular movements increase visibility of *Daphnia* to predators(14). Changes in *Daphnia* swimming behavior have been found with toxin exposure(15). In this experiment, we examined swimming behavior to determine if nanoparticles have a sublethal impact on this behavior. If so, this could have a larger impact on food web dynamics.

Lastly, feeding behavior was analyzed. *Daphnia* as filter-feeders are part of the most important groups of primary consumers in aquatic environments(16). Alterations in feeding rates of *Daphnia* can be an indicator of larger ecosystem effects. Feeding behavior is a key component in *Daphnia* survival. Toxicant-induced changes in feeding behavior allow for rapid estimate of the effects of a contaminant on individuals(11) that can have larger implications on survival. We measured the feeding appendage movement rate in addition to movement of the abdominal claw which is used by the organism to clean the thoracic legs (feeding appendages) of unwanted material(17) and may be an indicator of nanoparticle accumulation on the feeding appendages.

Hopping and heart rate as well as feeding behavior were examined with respect to exposure to fullerenes (nano-C₆₀), hydrogenated fullerenes (C₆₀HxC₇₀Hx), and titanium dioxide (TiO₂). Each nanoparticle type was chosen because of its current and potential uses in technology such as medicine, sporting equipment, cosmetics, coatings, and fuel cells(18). TiO₂ has been used in solar energy cells(19) and is being examined for use as an antitumor agent(20). Fullerenes and their derivatives are being developed for semiconductors and energy storage(21) as well as unique applications such as electronic paper(22). We predicted that because of the distinctive attributes of each nanoparticle type and the variation in toxicity seen in other studies that organisms would exhibit different physiological and behavioral responses to each particle type and functional group.

EXPERIMENTAL

Exposure to nanoparticles

Daphnia magna were kept according to U.S. EPA standard operating procedure (23) in a 17° Celsius incubator with 12-hour light and 12-hour dark cycle. Nanoparticles were prepared and characterized according to Lovern and Klaper (2006). Briefly, because carbon particles are typically hydrophobic, 20mg of each type of nanoparticle (99.5% purity) (Alfa Aesar, Ward Hill, MA, U.S.A.) were placed into 200mL of tetrahydrofuran (THF), sparged with nitrogen, and left to stir overnight. The solution was then filtered with a 0.22-mm nylaflo filter (Gelman Sciences, Ann Arbor, MI, U.S.A.), and 200mL of deionized water added. The THF was evaporated with a Büchi rotovapor (Büchi Labortechnik, Flawil, Switzerland) and an equal volume of deionized water added and evaporated twice more. Lastly, the solutions were once again filtered through a 0.22-mm nylaflo filter. Particle suspensions were characterized using Transmission Electron Microscopy was used to determine particle concentration and size (3).

Recently, debate about the preparation of nanoparticles and its effect on toxicity of the suspension has occurred. THF was used in this experiment in order to attain particles in the size-range of 10–20nm. For fullerene suspensions, solvent selection will affect the formation and size of particle aggregates as well as charge strength(24). Use of THF in sample preparation may cause nanoparticles to have greater negative ionic charge(25). However, the organic solvent is largely removed in the formation of aqueous suspensions of nano-C₆₀(24). In both carbon suspension and TiO₂ used in this experiment, UV-Vis spectroscopy showed no distinguishable peaks of THF. Additionally, the LD50 for THF in *Daphnia* is 5930ppm (26), which is over 20 times greater than the levels of nanoparticles present in this experiment. Furthermore, this type of sample preparation accurately represents particle preparation for scientific and industrial purposes (27,28).

The nanoparticles in the carbon suspensions averaged 10 to 20 nm in diameter, whereas TiO₂ had an average particle diameter of 30 nm. Both nano-C₆₀ and TiO₂ were prepared at the LOEC (Lowest Observable Effect Concentration) for the behavior tests, 260ppb and 2.0ppm respectively (3). As mortality results had yet to be determined, C₆₀HxC₇₀Hx was tested at the LOEC concentrations of C₆₀ as a comparison of impact of this particle at the same concentration.

In order to describe minute behavior and physiological changes, a minimum of six *Daphnia* were exposed to each treatment including a control of MHRW. *Daphnia magna* were tethered to a squirrel hair using Scotch-weld instant adhesive (3M, St. Paul, MN, USA). This hair was attached to a bendable wire attached to a 20cm wooden stick. The animal was allowed to acclimate at least 45 minutes to the tether prior to testing(29). A filming vessel containing 100ml of moderately hard reconstituted water (MHRW)(30) was placed in the light beam of a compound microscope mounted at a 90° angle. A Fastcam Super 10K high-speed camera (Photron, San Diego, CA, U.S.A.) recorded the image at 250 frames per second for 8.7 second intervals throughout the 2.5 hour duration of the experiment. Three clips were captured at each fifteen minute interval and then downloaded to an S-VHS tape for analysis.

Pre-exposure baseline rates of each behavior were obtained for 30 minutes prior to adding nanoparticles. Nanoparticles were then added to the vessel with a pipette and the animal was recorded for 1 hour (Fig 1). Time zero is the beginning of exposure immediately following the introduction of nanoparticles with a pipette. Prior observations adding ink with a pipette showed that the solutions diffuse rapidly throughout the container with this delivery method. After 60 minutes of exposure, the water was exchanged and fresh MHRW was used as replacement.

Each video segment was analyzed for heart rate, appendage and postabdominal curling rate, and hopping frequency over time. Heart rate was quantified by the number of contractions viewed per second. A full rotation of the first thoracic leg was used to measure feeding rate. Hopping was exhibited by the downward thrusting of the second antennae below the helmet and then back above. Last, a postabdominal tail curl was quantified when the postabdominal claw was brought proximally towards the thoracic appendages.

Statistics

An ANOVA repeated measures test (SPSS, version 13; SPSS Inc., Chicago, IL, U.S.A.) was used to evaluate if each time point played a role in behavioral changes over the duration of the experiment. Every time category (prior to, during exposure, and post-exposure) was compared within the category as well as among categories. A Tukey's Post Hoc test examined the variation of each behavior from one another and the control ($P < 0.10$) for each particle treatment.

RESULTS and DISCUSSION

Changes in Hopping Frequency with Nanoparticle Exposure

$C_{60}HxC_{70}Hx$ had the greatest impact on hopping frequency of the exposed *Daphnia* (Table 1). *Daphnia* in the $C_{60}HxC_{70}Hx$ suspension increased hopping by an average of 121 hops per minute ($\bar{X}_{prior} = 62.20 \pm 13.36$, $\bar{X}_{exposure} = 183.52 \pm 29.47$) and were statistically different from the controls during exposure ($P_{Hop} < 0.001$). Upon exposure to nano- C_{60} , the hopping frequency increased by an average of 113 additional hops per minute ($\bar{X}_{prior} = 47.89 \pm 5.75$, $\bar{X}_{exposure} = 161.06 \pm 12.13$) and was also statistically significant when compared to the control ($P_{Hop} < 0.001$) (Fig 1A). TiO_2 -exposed *Daphnia* had no significant change in hopping frequency (-5.9 hops per minute over the prior to exposure rate) and were not different from the control ($P_{Hop} = 0.892$). Additionally, there was a significant difference between TiO_2 -exposed and nano- C_{60} treatments ($P < 0.001$) and TiO_2 -exposed and $C_{60}HxC_{70}Hx$ treatments ($P_{Hop} < 0.001$).

Changes in Heart Rate with Nanoparticle Exposure

Nano- C_{60} was the only suspension to cause a significant change in heart rate from the control ($P_{HR} < 0.001$), increasing the average rate by 43.6 beats per minute ($\bar{X}_{prior} = 317.09 \pm 7.21$, $\bar{X}_{exposure} = 360.78 \pm 4.92$) (Fig 1B). $C_{60}HxC_{70}Hx$ caused an average decrease of -4.37 beats per minute and was not statistically significant ($P_{HR} = 0.806$). The decrease in heart rate for TiO_2 was not statistically significant ($P_{HR} = 0.101$) when compared to the control as the average decrease of each organism was only -1.66 . The nano- C_{60} treatment heart rate was also greater than TiO_2 and $C_{60}HxC_{70}Hx$ treatments ($P_{HR} < 0.001$ for both).

Changes in Appendage Movement with Nanoparticle Exposure

Appendage movement for the organisms exposed to nano- C_{60} increased by 64.51 cycles per minute when compared to the prior rate ($\bar{X}_{prior} = 300.69 \pm 5.03$, $\bar{X}_{exposure} = 365.20 \pm 7.95$) and this was statistically significant from the control ($P_{App} = 0.002$) (Fig 1C). *Daphnia* exposed to $C_{60}HxC_{70}Hx$ also increased appendage movement by 61.66 cycles per minute ($\bar{X}_{prior} = 326.69 \pm 10.73$, $\bar{X}_{exposure} = 388.35 \pm 30.95$) which was statistically different than the control ($P_{App} < 0.001$). The TiO_2 -exposed *Daphnia* did not significantly increase feeding appendage movement ($+8.76$) ($P_{App} = 0.116$).

Changes in Postabdominal Claw Curling with Nanoparticle Exposure

There were no significant changes in postabdominal claw curling for any of the exposure. Nano- C_{60} exposed *Daphnia* increased by 0.72 curls per minute ($P_{Pac} = 0.979$) and

C₆₀HxC₇₀Hx exposed increased by 0.38 curls per minute ($P_{\text{Pac}}=0.564$) (Fig 1D) compared to the control during the exposure period. TiO₂ exposed had the largest change (−1.28) but this was still not statistically significant from the control ($P_{\text{Pac}}=0.172$).

Recovery Rate after Nanoparticle Exposure

In general, exposed *Daphnia* required thirty minutes to completely return to the rates measured prior to exposure. This indicates that prolonged effects of particle exposure occurred in comparison to the controls that recovered to baseline rates after the initial shock of water transfer. However, the nano-C₆₀-exposed *Daphnia* did not return to pre-exposure levels during the sixty-minute recovery period ($P_{\text{Hop}} < 0.001$, $P_{\text{App}} = 0.010$, $P_{\text{HR}} < 0.001$) (Table 1 and Fig 2). Conversely, C₆₀HxC₇₀Hx-exposed *Daphnia* did recover to pre-exposure rates for feeding, indistinguishable from pre-exposure rates ($P = 0.392$). *Daphnia* exposed to TiO₂ remained indistinguishable from the control during the treatment and recovery period.

Throughout the exposure period, behaviors were not significantly different over time ($P_{\text{HR}} = 0.697$, $P_{\text{App}} = 0.777$, $P_{\text{Hop}} = 0.225$, $P_{\text{Pac}} = 0.433$). However, time was a significant component in the recovery period for heart rate ($P_{\text{HR}} < 0.001$) with a return to pre-exposure levels after fifteen minutes. Also, hopping frequency ($P_{\text{Hop}} = 0.001$) over time decreased with TiO₂-exposed *Daphnia magna*, but due to the large standard error, the rate in appendage beat frequency was not significant ($P_{\text{App}} = 0.521$).

Influence of Nanoparticle suspension on Behavior

The results of this study suggest that nanoparticles not only affect mortality in *Daphnia magna* (3), but also have sublethal effects in the form of behavioral changes. Additionally, the type of nanoparticle and functional group plays an important role in determining the effect on the animal. *Daphnia* exposed to solutions of nano-C₆₀ and C₆₀HxC₇₀Hx showed the most drastic changes in hopping frequency, appendage movement, and heart rate, while TiO₂ solutions caused no statistically significant changes in any measures.

Hopping rate

Hopping rate was affected by both types of carbon compounds, nano-C₆₀ and the hydrogenated C₆₀HxC₇₀Hx. However, only the *Daphnia* exposed to C₆₀HxC₇₀Hx returned to pre-exposure hopping frequency during the recovery period. Hopping is important as it is a characteristic of avoidance and escape response for *Daphnia*. Escape response is elicited in *Daphnia* when exposed to predators(31) or the kairomones of predators(12) but is also an indicator of avoidance of toxins(32). Avoidance behavior is an early and sensitive indicator of environmental state and provides a more detailed assessment of impact of contamination to compliment traditional techniques(7,32).

Each swimming behavior of *Daphnia magna* affects the risk of mortality due to predation(8), and as the exposure to nanoparticles changed this behavior, *D. magna* could become more obvious to predators due to the presence of nanoparticles in aquatic systems. Increasing hopping rate might cause the *D. magna* to be more apparent to visual predators, causing predation rates to increase. Furthermore, if the nanoparticles bioaccumulate in the *D. magna*, then an increase in predation would result in greater biomagnification in the food web as is suggested for pesticide-exposed zooplankton(6). In addition, bioaccumulation could cause particles originally in small concentrations to accumulate to lethal concentrations at higher trophic levels.

Lastly, because predation defense is thought to be energetically costly, it is usually employed only when necessary(33). Increases in escape behavior caused by carbon-based nanoparticle exposure increase energy expenditure and could lower growth and reproduction rates.

Reduction in the growth rate has been shown to cause maturation at smaller sizes and production of less offspring per brood(6).

Heart rate

Changes in behaviors like those of respiration rates occurring at sublethal concentrations of toxic substances may inhibit population growth and have long-term negative impacts on a community(11). *Daphnia* heart rate is known to increase with low oxygen levels brought on by anoxic conditions(34) or high temperature(9). When the ambient oxygen partial pressure decreases, *Daphnia* heart rate increased(35). Our results indicate that only the nano-C₆₀ treatment increased the heart rate while the other suspensions had no effect. During exposure period, nano-C₆₀ caused an average increase of 43.6 beats per minute. C₆₀HxC₇₀Hx did not show a difference in heart rate, so it appears that even if minimal amounts of THF remain in solution, they are not impacting this characteristic. Evaluating the change in heart rate will help in understanding the differences exhibited in physiological changes upon exposure to various nanoparticles.

Feeding and Postabdominal Curling Rates

In addition to effects on hopping frequency, feeding appendage movement was also increased by exposure to nano-C₆₀. There are several possibilities as to why this may have occurred. *Daphnia* consume large quantities of algae by creating a current of water that runs through their carapace by beating the thoracic legs rhythmically(17). From the feeding column created by this current, the *Daphnia* will pick which items to ingest, selecting particles on the basis of size, shape, and texture(36). *Daphnia* decrease feeding rates when exposed to low levels of food(37) and this reduction in feeding may cause a reduction in growth and reproduction dynamics(6). The nano-C₆₀ exposed *Daphnia* may be increasing their appendage rate to obtain more particles, mistaking them as food.

Conversely, the *Daphnia* may actually be moving the column of water through its appendages to rid itself of particles. If the organism is, in fact, sensing the toxicant in the solution and rejecting the water, it may increase the rate of filtration in hopes of bringing fresh, nanoparticle-free water into its carapace. If this is the case, it is likely that the *Daphnia* would swim away from the region if it were not tethered. Abnormal swimming was exhibit in our initial study (3) and should be investigated further to see if avoidance behaviors similar to that found by Lopes *et al.* (2004) are occurring(7,32).

However, if the nano-C₆₀ solution is interfering with the sensory ability of the *Daphnia*, this could cause the system to react inappropriately. Contaminants such as crude oil(29), sodium dodecyl sulphate (SDS)(11), and pesticides(6) cause an immediate decrease in feeding behavior of *Daphnia*, even at sub-lethal levels. However, the appendage movement exhibited in this study actually increased in appendage beat frequency. If the nanoparticles are interfering with sensory response, the proposed advantageous response of decreased feeding does not occur and excess energy is used in increasing filtration rates. This loss in energy would have negative impacts on growth and reproduction(6).

There was no change in postabdominal curling. This behavior might increase if nanoparticles were clogging the feeding appendages. However, due to their minute size, even if the nanoparticles did accumulate on the organisms legs, they may go undetected. Other studies have suggested that nanoparticles accumulate on the carapace and appendages of zooplankton (38). Under these circumstances, increases in activity may correspond to *Daphnia* attempting to clean the particles off of their appendages; however, if this were the case, an increase in postabdominal curling would be expected. Additionally, SEM images we have taken showed

no accumulation of nanoparticles on the appendages. The ability of *Daphnia* to ingest these types of nanoparticles is still unknown.

Summary

Results from this experiment suggest that the functional groups may have a drastic effect on the sublethal effects of nanoparticles on behavior. This is especially noticeable from the ability of the *Daphnia* to recovery to pre-exposure behavior levels when exposed to C₆₀HxC₇₀Hx but not the suspension of nano-C₆₀. Sayes *et al.* (2004) and Chen *et al.* (2006) showed that cytotoxicity of nanoparticles is affected by addition of functional groups. Furthermore, functional groups and sites of attachments determine the effectiveness of antifouling agents against barnacle settlement(39). When various compounds were synthesized with differing isocyano functional groups, those with acetyl esters proved most affective in preventing barnacle attachment(39,40). Sato et al (2005) also noted that functional group modification affected the cytotoxicity of H-CNFs, affecting cell activation(41). Hydrogenation of the C₆₀HxC₇₀Hx suspension may be one of the reasons that it did not show a change in heart rate and the *Daphnia* exhibited intermediate responses of the other behaviors.

With the knowledge of the effect of nanoparticles on behavior, the question arises as to if these traits will occur in aquatic environments. As the use of nanoparticles in manufactured goods increases(4,42,43), exposure becomes more likely. Just as pesticides reduce survivorship of zooplankton by controlling prey behavior even at sublethal levels(6), we have shown that nanoparticle suspensions also alter *Daphnia* behavior at sublethal concentrations. Filter feeding is the most important trophic interface of zooplankton with phytoplankton(37), so if *Daphnia* filtration rates are altered, this intricate balance could be at risk.

This experiment demonstrates the utility of behavioral assays in assessing the impact of three types of nanoparticles and uses behavioral endpoints to better understand exposure to nanoparticles at sublethal levels. Although the experimental set-up is fairly involved, the repeatability and ability to gauge small-scale behavioral changes allows for intricate behaviors to be deciphered. Behavioral changes in *Daphnia* may affect reproduction, predation, and food intake, as well as food web interactions. Given the importance of *Daphnia* to ecosystems, the release of nanoparticles could be detrimental to aquatic environments even at low concentrations and would be highly dependent upon particle type.

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References

1. The United Nations Educational, Scientific, and Cultural Organization. The Ethics and Politics of Nanotechnology. UNESCO; 2006.
2. Chapman PM. Emerging Substances-Emerging Problems? Environmental Toxicology and Chemistry 2006;25:1445–1447. [PubMed: 16764460]
3. Lovern SB, Klaper R. *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C₆₀) nanoparticles. Environmental Toxicology and Chemistry 2006;25:1132–1137. [PubMed: 16629153]
4. Cagle DW, Kennel SJ, Mirzadeh S, Alford JM, Wilson LJ. *In vivo* studies of fullerene-based materials using endohedral metallofullerene radiotracers. Proceedings of the National Academy of Sciences of the United States of America 1999;96:5182–5187. [PubMed: 10220440]

5. Chen X, Tam UC, Czapinski JL, Lee GS, Rabuka D, Zettl A, Bertozzi CR. Interfacing Carbon Nanotubes with Living Cells. *Journal of the American Chemical Society* 2006;128:6292–6293. [PubMed: 16683774]
6. Hanazato T. Pesticide effects on freshwater zooplankton: an ecological perspective. *Environmental Pollution* 2001;112:1–10. [PubMed: 11202648]
7. Yearley RB Jr, Lazorchak JM, Gast LC. The potential of an earthworm avoidance test for evaluation of hazardous waste sites. *Environmental Toxicology and Chemistry* 1996;15:1532–1537.
8. O'Keefe TC, Brewer MC, Dodson SI. Swimming behavior of *Daphnia*: its role in determining predation risk. *Journal of Plankton Research* 1998;20:973–984.
9. Lamkemeyer T, Zeis B, Paul RJ. Temperature acclimation influences temperature-related behaviour as well as oxygen-transport physiology and biochemistry in the water flea *Daphnia magna*. *Can. J. Zool* 2003;81:237–249.
10. Campbell AK, Wann KT, Matthews SB. Lactose causes heart arrhythmia in the water flea *Daphnia pulex*. *Comparative Biochemistry and Physiology Part B* 2004;139:225–234.
11. Jones M, Folt C, Guarda S. Characterizing Individual, Population and Community Effects of Sublethal Levels of Aquatic Toxicants - an Experimental Case-Study Using *Daphnia*. *Freshwater Biology* 1991;26:35–44.
12. Brewer MC, Dawidowicz P, Dodson SI. Interactive effects of fish kairomone and light on *Daphnia* escape behavior. *Journal of Plankton Research* 1999;21:1317–1335.
13. Zaret, TM. The Effect of Prey Motion on Planktivore Choice. In: Kerfoot, WC., editor. *Evolution and Ecology of Zooplankton Communities*. Hanover, NH: The University Press of New England; 1980. p. 594–603.
14. Strickler JR, Udvardi AJ, Marino J, Radabaugh N, Ziarek J, Nihongi A. Visibility as a factor in the copepod - planktivorous fish relationship. *Sci. Mar* 2005;69:111–124.
15. Baillieul M, Blust R. Analysis of the swimming velocity of cadmium-stressed *Daphnia magna*. *Aquatic Toxicology* 1999;44:245–254.
16. Gerritsen J, Porter KG. The Role of Surface Chemistry in Filter Feeding by Zooplankton. *Science* 1982;216:1225–1227. [PubMed: 17830583]
17. Thorp, JH.; Covich, AP., editors. *Ecology and Classification of North American Freshwater Invertebrates*. Second Edition ed. San Diego: Academic Press; 2001.
18. Morris, J.; Willis, J.; Gallagher, K., editors. *Nanotechnology White Paper*. Washington, D.C.: U.S. Environmental Protection Agency; 2005. p. 134
19. Shipway AN, Katz E, Willner I. Nanoparticle Arrays on Surfaces for Electronic, Optical, and Sensor Applications. *Chemphyschem* 2000;1:18–52.
20. Cai R, Kubota Y, Shuin T, Sakai H, Hashimoto K, Fujishima A. Induction of cytotoxicity by photoexcited TiO₂ particles. *Cancer Research* 1992;52:2346–2348. [PubMed: 1559237]
21. Baughman RH, Zakhidov AA, Heer WAd. Carbon Nanotubes-The Route Toward Applications. *Science* 2002;297:787–792. [PubMed: 12161643]
22. Huitema E, Gelinck G, van Lieshout P, van Veenendaal E, Touwslager F. Flexible electronic-paper active-matrix displays. *Journal of the Society for Information Display* 2005;13:181–185.
23. SoBran Inc.. *Standard Operating Procedure for Mass Culture (*Daphnia pulex*)*. Cincinnati, OH: U.S. Environmental Protection Agency; 2004. p. 1–3.
24. Brant JA, Labille J, Bottero JY, Wiesner MR. Characterizing the Impact of Preparation Method on Fullerene Cluster Structure and Chemistry. *Langmuir* 2006;22:3878–3885. [PubMed: 16584270]
25. Brant J, Lecoanet H, Hotze M, Wiesner M. Comparison of Electrokinetic Properties of Colloidal Fullerenes (*nC₆₀*) Formed Using Two Procedures. *Environ. Sci. Technol* 2005;39:6343–6351. [PubMed: 16190186]
26. Sigma-Aldrich. *Material Data Safety Sheet-Tetrahydrofuran*. Sigma-Aldrich; 2003.
27. Kim DH, Lee H, Song CK, Lee C. Fabrication of nanosized molecular array device and logic gate using dimethyl-phenylethynyl thiol. *Journal of Nanoscience and Nanotechnology* 2006;6:3470–3473. [PubMed: 17252791]
28. Higuchi T, Yabu H, Shimomura M. Simple preparation of hemispherical polystyrene particles. *Colloids and Surfaces a-Physicochemical and Engineering Aspects* 2006;284:250–253.

29. Wong C, Strickler J, Engelhardt F. Feeding behavior of *Daphnia pulex* in crude oil dispersions. *Bulletin of environmental contamination and toxicology* 1983;31:152–157. [PubMed: 6616084]
30. SoBran Inc.. Standard Operating Procedure for Moderately Hard Reconstituted Water, #204. Cincinnati, OH: U. S. Environmental Protection Agency; 2003. p. 1-5.
31. Seely CJ, Lutnesky MMF. Odour induced antipredator behaviour of the water flea, *Ceriodaphnia reticulata*, in varying predator and prey densities. *Freshwater Biology* 1998;40:17–24.
32. Lopes I, Baird DJ, Ribeiro R. Avoidance of copper contamination by field populations of *Daphnia longispina*. *Environmental Toxicology and Chemistry* 2004;23:1702–1708. [PubMed: 15230322]
33. Tollrian, R.; Harvell, CD. *The Ecology and Evolution of Inducible Defenses*. Princeton, New Jersey: Princeton University Press; 1999.
34. Paul RJ, Colmorgen M, Pirow R, Chen YH, Tsai MC. Systemic and metabolic responses in *Daphnia magna* to anoxia. *Comparative Biochemistry and Physiology Part A* 1998;120:519–530.
35. Baumer C, Pirow R, Paul RJ. Circulatory oxygen transport in the water flea *Daphnia magna*. *J. Comp. Physiol. B* 2002;172:275–285. [PubMed: 12037590]
36. Porter KG. The plant-animal interface in freshwater ecosystems. *American Scientist* 1977;65:159–170.
37. Thompson J, Ferguson J, Reynolds C. Natural filtration rates of zooplankton in a closed system: the derivation of a community grazing index. *Journal of plankton research* 1982;4:545–560.
38. Ferguson, P.; DeMarco, A.; Templeton, R.; Chandler, G. SETAC North America 26th Annual Meeting; Baltimore, MD. 2005.
39. Kitano Y, Nogata Y, Shinshima K, Yoshimura E, Chiba K, Tada M, Sakaguchi I. Synthesis and anti-barnacle activities of novel isocyanocyclohexane compounds containing an ester or an ether functional group. *Biofouling* 2004;20:93–100. [PubMed: 15203963]
40. Kitano Y, Yokoyama A, Nogata Y, Shinshima K, Yoshimura E, Chiba K, Tada M, Sakaguchi I. Synthesis and anti-barnacle activity of novel 3-isocyanothionellin analogues. *Biofouling* 2003;19:187–192. [PubMed: 14618719]
41. Sato Y, Shibata K, Kataoka H, Ogino S, Bunshi F, Yokoyama A, Tamura K, Akasaka T, Uo M, Motomiya K, Jeyadevan B, Hatakeyama R, Watari F, Tohji K. Strict preparation and evaluation of water-soluble hatstacked carbon nanofibers for biomedical application and their high biocompatibility: influence of nanofiber-surface functional groups on cytotoxicity. *Molecular Biosystems* 2005;1:142–145. [PubMed: 16880976]
42. Sayes CM, Fortner JD, Guo W, Lyon D, Boyd AM, Ausman KD, Tao YJ, Sitharaman B, Wilson LJ, Hughes JB, West JL, Colvin VL. The Differential Cytotoxicity of Water-Soluble Fullerenes. *Nano letters* 2004;4:1881–1887.
43. Foley S, Crowley C, Smaih M, Bonfils C, Erlanger BF, Seta P, Larroque C. Cellular localisation of a water-soluble fullerene derivative. *Biochemical and Biophysical Research Communications* 2002;294:116–119. [PubMed: 12054749]

Figure 1.

Figure 1 shows the average change in behavior (events/minute with standard error) for each treatment group during the 60-minute exposure. Time zero is the beginning of exposure immediately following the introduction of nanoparticles with a pipette. Time 60 is the end of the exposure period. Each behavior (shown on the y axis) is the number of the events per minute. Each treatment is displayed as follows: Control (Δ), TiO₂ (▼), Nano-C₆₀ (○), C₆₀HxC₇₀Hx (●). ANOVA analysis showed hopping frequency was significantly altered in nano-C₆₀ ($P < 0.001$) and C₆₀HxC₇₀Hx ($P < 0.001$) suspensions. Heart rate was significantly increased in only the nano-C₆₀ suspension ($P < 0.001$). Appendage movement was significantly increased in nano-C₆₀ ($P = 0.002$) and C₆₀HxC₇₀Hx ($P < 0.001$). However, postabdominal curling was not affected by any of the treatments.

Figure 2.

Figure 2 shows the average change in behavior (events/minute with standard error) for each treatment group during the 60-minute recovery period after exposure. Time zero is the beginning of exposure immediately following the introduction of nanoparticles with a pipette. Time 60 is the end of the exposure period. Each behavior (shown on the y axis) is the number of the events per minute. Each treatment is displayed as follows: Control (Δ), TiO₂ (▼), Nano-C₆₀ (○), C₆₀HxC₇₀Hx (●). Nano-C₆₀ exposed *Daphnia* did not return to pre-exposure levels during the recovery period for hopping, appendage, or heart rate (Tukey's Post Hoc test; $P_{\text{Hop}} < 0.001$, $P_{\text{App}} = 0.010$, $P_{\text{HR}} < 0.001$). *Daphnia* exposed to C₆₀HxC₇₀Hx did return to rates indistinguishable from the control.

Table 1

Average rate of each behavior during all time points during the three treatments, prior to exposure, during exposure, and post-exposure with standard error

	Hopping Rate			Heart Rate		
	Prior	Exposure	Post	Prior	Exposure	Post
Control	43.93±5.47	38.47±6.93	35.73±5.47	309.83±5.84	307.74±6.41	310.34±9.83
nano-C ₆₀	47.89±5.75	161.06±12.13	145.11±9.83	317.09±7.21	360.78±4.92	355.50±4.32
C ₆₀ HxC ₇₀ Hx	62.20±13.36	183.52±29.47	61.02±9.36	304.60±3.88	300.23±5.82	309.20±6.16
TiO ₂	58.36±12.97	52.41±9.95	50.29±9.01	328.86±9.07	327.20±11.95	317.82±7.01
Appendage Beat						
Post-abdominal Curl						
Control	278.93±8.39	276.01±9.36	294.51±4.81	6.39±1.31	5.90±1.34	5.56±1.40
nano-C ₆₀	300.69±5.03	365.20±7.95	360.88±7.01	5.59±0.96	6.25±1.19	5.29±1.21
C ₆₀ HxC ₇₀ Hx	326.69±10.73	388.35±30.95	317.15±14.44	4.21±1.30	4.60±1.27	5.17±1.30
TiO ₂	327.71±18.64	336.48±27.39	366.30±41.97	5.11±1.59	3.83±1.396	2.49±0.92