Chapter 5

Ecotoxicity of Fullerenes and Carbon Nanotubes: A Critical Review of Evidence for Nano-Size Effects

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The promise of nanotechnology is expected to impact almost every field with widespread incorporation of nanoparticles (NPs) in numerous commercial products. While the unique properties of NPs and their applications offer important benefits, some concerns have been raised such as the potential for NPs to pose unique risks to human and environmental health upon release into the environment. Initial speculation of novel toxicities from NPs needs to be reevaluated based on actual evidence from ecotoxicological exposure studies. In this chapter, we review the literature on ecotoxicity of fullerenes (C_{60}) and carbon nanotubes in multi-cellular organisms and evaluate the evidence for toxicological effects to be a consequence of the nano-size of these NPs. We find that absorption of these NPs and their entrance into systemic circulation has not been observed in the few studies that have investigated biodistribution in organisms under environmentally relevant conditions, and where tissue concentrations have been determined, they are exceedingly low. Limited absorption of these NPs into organisms suggests that toxicological effects reported in internal tissues should be interpreted cautiously

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and not presumed to be a nano-size effect from these NPs. Experimental artifacts such as the use of vehicle solvents (e.g., tetrahydrofuran) appear to account for the majority of the highly toxic effects observed for fullerenes. At the present time, there is not sufficient evidence to conclude a nano-size toxicological effect for C_{60} within the ecotoxicology literature for multi-cellular organisms, but there are some effects from carbon nanotubes that may be attributable to nano-size effects.

Introduction

Nanotechnology has been described as a scientific revolution with future applications expected to transform a broad range of fields. The small particle size of nanoparticles (NPs), which are defined as having one dimension within the range of 1 to 100 nanometers, often yields exciting new properties that substantially differ from bulk particles of a similar chemical composition. With NPs already being incorporated into numerous consumer products and many more usages expected in upcoming years (for the full current list of consumer products containing engineered nanomaterials (ENs), it is suggested that the reader visit the following Web site: http://www.nanotechproject.org/inventories/consumer/), one major concern is to what extent NPs may pose environmental or human health risks (*1–3*). New technologies often have unexpected consequences and the nanotechnology revolution is not expected to be an exception. However, what is largely different about nanotechnology is that proactive research is being conducted to assess their potential risks, if any, *a priori*.

It has been postulated that nanoparticles will likely cause elevated risks as a result of their small size (*2*), which is often referred to as the so-called "nano" effect. The likelihood for heightened risks from these materials has been hypothesized to stem from their increased surface area and reactivity and the higher particle numbers for a similar mass when compared to bulk materials. While preventing unexpected risks to humans and ecological receptors is an important motivating factor for studies about the potential risks of nanoparticles, there are also serious risks to overstating results or speculating about the risks of nanoparticles without sufficient scientific evidence. Results indicating toxic effects from nanomaterials may spread fears about nanotechnology throughout the public, and later scientific evidence may not be able to sway these initial opinions. While a precautionary principle is prudent regarding toxicity of nanomaterials, overstated or unrealistic results of toxicity for a particular NP could generate negative perceptions of the nanotechnology industry and limit future benefits (*4*). This would certainly have a chilling effect on technological advancements related to nanotechnology, advancements which could have otherwise led to a substantial positive impact on our standard of living. On the other hand, it is cavalier to assume that there will be no risks from NP exposure or release into the environment, and the development of nanotechnology without consideration of the potential harmful effects could have serious negative impacts on human and environmental well-being. There is a need to understand and

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avoid the potential risks associated with nanotechnology, and, at the same time, to avoid overstatement of such risks that could impede realization of the full benefits of the technology. Such a balance requires the usage of environmentally realistic exposure conditions to build a solid scientific foundation related to the environmental behaviors and risks of NPs.

The extent to which early predictions about elevated "nano" risks to organisms have held true was recently examined for metal NPs and it was suggested that particles larger than 30 nm typically do not have a different toxic effect compared to bulk particles (*5*). However, this trend has not yet been evaluated, to our knowledge, for carbon nanoparticles even though some consumer goods already utilize these nanoparticles. The purpose of this chapter is to review the current scientific literature to assess to what extent the potential risks for carbon nanotubes and fullerenes, two classes of carbon nanomaterials, pose novel risks to multi-cellular organisms in the environment as a result of their nano-scale size. Methodological considerations and potential experimental artifacts unique to NPs in general and these NPs in particular will be highlighted, and an overall assessment of the evidence for nano-size effects for these materials will be provided.

Background for Carbon Nanotubes and Fullerenes

Carbon Nanotubes

Carbon nanotubes (CNTs), first discovered by Iijima in 1991 (*6*), comprise one of the most promising classes of new materials to emerge from nanotechnology to date. Their unique structure, composed of extensive sp^2 carbons arranged in fused benzene rings, provides exceptional material properties with respect to electrical and thermal conductivity, strength, and high surface-to-mass ratios. These characteristics in turn make them suitable for numerous potential applications, including uses in composite materials, sensors, hydrogen-storage fuel cells, and various environmental applications (*7–10*). Two principal types of carbon nanotubes have been fabricated: single-walled carbon nanotubes (SWNTs), which are one-layered graphitic cylinders having diameters on the order of a few nanometers, and multi-walled carbon nanotubes (MWNTs), which comprise between 2 to 30 concentric cylinders having outer diameters commonly between 30 to 50 nm. One special type of MWNTs that has received substantial research attention is double-walled carbon nanotubes (DWNTs), which are composed of two concentric cylinders. For a more extensive discussion of the unique properties and characteristics of carbon nanotubes, please see a recent review (*10*).

Fullerenes

Carbon molecules arranged into a spherical shape resembling a geodesic dome have become known as fullerenes in honor of the visionary American architect R. Buckminster Fuller that designed prominent buildings of this configuration (*11*). Although carbon molecules can be arranged into different spherical configurations involving different numbers of carbon atoms (e.g., C_{60},

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C_{70}, C_{80}, etc.), the C_{60} (buckminster fullerene or "Bucky ball") is by far the most prominent in terms of production, scientific interest, and research engagement. Considerable interest and speculation has surrounded the C_{60} fullerene since first preparation of the nanoparticle was achieved in the laboratory (*12*), and this speculation has predicted both beneficial uses (*11*, *13*, *14*) as well as unexpected negative consequences (e.g., toxicity after C_{60} release into the environment) (*1*). The elegant configuration of sixty carbon atoms into a spherical arrangement confers unique physicochemical properties to C_{60}, which have been reviewed in detail in numerous publications (*15–17*). Partially de-localized π-electrons in C_{60} can absorb energy (e.g., light) and can promote formation of reactive oxygen species (ROS) (*18*). The potential for large increases in production of C_{60} (e.g., for use in consumer products), consequent releases into the environment, and possible C_{60}-induced toxicity in organisms including humans has led to numerous recent research investigations into the environmental implications of this nanoparticle (*19*). However, studies of the environmental fate and toxicity of C_{60} are limited by a lack of established scientific methods for evaluation of the behavior of C_{60} in environmental media and for testing toxicity of C_{60} in an environmentally relevant context.

Investigations of the Toxicity of Carbon Nanotubes

This section is divided roughly into terrestrial, sediment, and aquatic (i.e., no sediment) ecosystems. This review is intended to provide a brief overview of the literature to assess to what extent nano-size toxic effects have been observed for carbon nanotubes.

Sediment

The toxicity and bioaccumulation of carbon nanotubes in sediment ecosystems has been investigated in six studies (*20–25*). In the four studies that investigated to what extent single- or multi-walled carbon nanotubes would accumulate in organisms, researchers found negligible absorption into the organism tissues of oligochaetes (*24*, *25*), two estuarine invertebrates (*20*), and a lugworm (*21*). Changing the properties of the carbon nanotubes so that they possess higher octanol-water distribution coefficients, a change that typically corresponds with higher organism accumulation for hydrophobic organic chemicals (HOCs), was not found to increase their bioaccumulation factor (BAF) values for the oligochaete *Lumbriculus variegatus* (*25*). Given the lack of absorption into organism tissues, it is important to differentiate between carbon nanotubes inside the gut tract of the organism and those absorbed into systemic circulation. The term accumulation is hereafter used to refer to the total mass of the carbon nanoparticle in the organism while uptake specifically refers to absorption across epithelial membranes and into internal tissues. The presence of SWNTs at a high sediment concentration of 5 mg/g was found to not change or decrease the uptake of a broad number of HOCs by two estuarine invertebrates (*20*). These results agree with modeling by Koelmans and coworkers who

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estimated that the presence of environmentally realistic concentrations of carbon nanotubes would not be expected to impact bioavailability of HOCs in sediment ecosystems (*26*).

Perhaps unsurprisingly in light of the lack of accumulation of nanotubes by organisms, sediments spiked with carbon nanotubes typically only had a minor ecotoxicological impact even when spiked at high concentrations. MWNTs and SWNTs spiked to sediments at concentrations of 0.3 and 0.03 g/kg did not cause increased oligochaete mortality after 28 days compared to control sediments (*24*). Similarly, spiking sediment with SWNTs at a concentration of 0.03 g/kg did not impact burrowing behavior, feeding rates, or DNA damage as measured by the comet assay for a lugworm (*21*). However, decreased survival was observed for *Hyallela azteca* and *Leptocheirus plumulosus* albeit at environmentally unrealistic concentrations of 300 g MWNT/kg sediment and 30 g/kg, respectively (*22*). While spiking MWNTs at a concentration of 99 g/kg did not have an impact on *H. azteca*, the lowest observed effect concentration was not investigated for *L. plumulosus*. It was also observed that the LC50 (i.e., concentration that is lethal to 50 % of the organisms) value for raw MWNTs using these same organisms was higher than that for activated carbon and carbon black (*23*). This suggests that MWNT toxicity may be less than that for activated carbon, an amendment that is being widely considered for treatment of contaminated sediments (*27*, *28*). Overall, the accumulation and toxicological results reported to date for sediment ecosystems do not indicate that these carbon nanotubes possess a uniquely elevated risk as a result of their nano-scale size.

Soil

There have been several studies on the effects of carbon nanotubes in earthworms (*25*, *29–31*). SWNTs and MWNTs did not accumulate within earthworms to significant extents even when the MWNTs were modified to be more hydrophilic (*25*, *29*). Additionally, both SWNTs and MWNTs were found to decrease earthworm uptake of pyrene, a polycyclic aromatic hydrocarbon, when the nanotubes were spiked to soils at concentrations of 3 g/kg, but not 0.3 g/kg (*30*). When considered in combination with prior experimental (*20*) and modeling (*26*) efforts, these results suggests that the presence of carbon nanotubes in the environment are expected to decrease HOC accumulation by organisms in a manner similar to that for black carbons when added at extremely high concentrations. The toxicity of DWNTs was also investigated using the earthworm *Eisenia veneta* (*31*). The most sensitive endpoint was reproduction as measured by earthworm cocoon production which was impacted at a food concentration above 37 mg DWNT/kg food, while survival and hatchability were not impacted at concentrations up to 495 mg DWNT/kg food. These results are not believed to result from metal catalysts associated with the DWNTs, but the toxicity mechanism was not determined. In a study by Petersen and coworkers (*30*), no effects were observed on the earthworm lipid content or dry mass after exposure to concentrations up to 3 g/kg for SWNTs and MWNTs in two soils, although this study was not specifically designed to test for subacute toxicity effects. Nevertheless, these results suggest that carbon nanotubes may cause

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sub-acute effects to organisms such as impacting their reproduction behaviors at relatively high concentrations, but that it is highly unlikely for them to impact the survival rates for adult organisms.

There have also been numerous studies on the effects of CNTs on plants conducted under hydroponic conditions (i.e., without soil). The effects of interactions with soil were not considered in any of these studies, although they would likely decrease the observed toxicity as a result of sorption/attachment interactions. Nevertheless, the observed effects of CNT exposure in plant species have been inconsistent. One study indicated that MWNT treatment did not impact plant germination for any of the plant species tested at an MWNT solution concentration of 2 g/L (*32*), while other studies showed decreases in root elongation for some plant species and increases for others after exposure to functionalized and non-functionalized SWNTs (*33*) or decreased biomass for *Cucurbita pepo* (zucchini) after MWNT exposure (*34*). The toxic effects observed for the zucchini appeared to be related to the properties of the dispersed carbon nanotubes, because activated carbon did not have this effect. Thus, this may be an effect related to the nano-sized structure of the carbon nanotubes. CNTs generally had a more pronounced effect on suspended plant cells (*32*, *35*) with MWNTs causing decreased cell viability and increasing reactive oxygen species at a concentration of 20 mg CNTs/L of medium (*35*). Carbon nanotubes showed an ability to pierce plant cells *in vivo* using two photon microscopy (*36*) but they did not fully enter the cells, and SWNTs did not appear to enter the roots of any plants when investigated by scanning electron microscopy (SEM) (*33*). These results agree with transmission electron microscope (TEM) micrographs of suspended rice cells exposed to MWNTs which showed MWNT contact with the cell wall but not internalization of the nanotubes (*35*). Raman spectroscopy showed uptake of MWNTs into tomato plant seeds, but they were not detectable in grown plant tissues (roots, leaves, or stems) (*37*). Overall, these results suggest that CNT internalization by plants will be limited. Additionally, some studies have indicated that carbon nanotubes had a positive effect on tomato plants enhancing germination rates and shortening the germination time (*37*), and non-functionalized SWNTs enhanced root elongation in onions and cucumbers (*33*). As such, the expected effects of carbon nanotubes on plant growth in hydroponic conditions are unclear and may vary based on the type of nanotube and plant species, yet some of these observed effects may be a result of the nano-sized structure of the CNTs. However, it is important to recognize that these effects may substantially differ in the presence of soil as would be typical for plant exposure in the natural environment. At a minimum, extreme caution is warranted in the usage of carbon nanotubes for agricultural products given the lack of a rigorous understanding about the risks these NPs could pose after ingestion.

Water

The majority of studies relating to the ecological impacts of carbon nanotubes have been conducted in water-only exposures. This may be in large part a result of the fact that detection of carbon nanotubes in matrices without soil or sediment is substantially easier. Unlike the studies conducted in soils and

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sediments, suspended carbon nanotubes have shown acute and sub-acute effects to organisms at low concentrations in the range of micrograms of nanotubes per liter of solution (i.e., (*38*)). Their toxicity has been investigated using a broad range of ecological organisms including fish (*39–42*), daphnia (*22*, *23*, *38*, *43–45*), estuarine copepods (*46*), amphibian larvae (*47*, *48*), protozoa (*49*), and bacteria (*50–53*). Despite the observed toxicity to these various organisms, there did not appear to be substantial absorption of MWNTs across the intestines by *Daphnia magna*, but rather a large mass of MWNTs appeared to be compacted in the organisms guts as measured microscopically and using radioactively labeled carbon nanotubes (*44*). Large masses of carbon nanotubes were also found in the guts in many other organisms using microscopic methods (*23*, *45*, *47*), but no paper to our knowledge has shown substantial absorption of carbon nanotubes across the gut linings in any aquatic organism.

As such, toxic effects from CNT exposure are expected to occur primarily in the digestive organs or gills, or after attachment to the surfaces of organisms, which could potentially influence their ability to swim as has been observed earlier for *Daphnia magna* exposed to fullerenes (*54*). Indeed, two studies with lipid-coated SWNTs and daphnia have suggested that the observed toxicity was likely a result of clumping and deposition in the organism intestines (*43*, *45*), which thus raises the question about whether the effects observed for NPs were a result of a nano-size effect or just suspended solid material that could deposit in the gut. Additionally, Kennedy and coworkers found that stirred MWNTs, which were more aggregated than sonicated MWNTs, were more toxic to *Ceriodaphnia dubia* than for sonicated nanotubes from the same source (*22*), a result which again contrasts with what would be expected for a nano-size toxic effect. One potential artifact which could be the cause of toxicity in studies with carbon nanotubes is the release of toxic metals from the catalyst materials. It was recently determined that yttrium released from carbon nanotubes affected the functioning of neuronal calcium channels (*55*). The impact of released metals was also suggested as a potential cause of the differing effects of SWNT and DWNT exposure on zebrafish embryos (*41*). It is important to note that broad differences were observed in the toxic impact of the CNTs on the various organisms. Larvae of the amphibian *Ambystoma mexicanum* did not exhibit increased mortality or genotoxicity after exposure to DWNTs at concentrations up to 1 g/L (*48*), while *Daphnia magna* had a 96-h LC50 value of 2.48 mg MWNTs/L (*38*). The cause for the substantially different sensitivities among these organisms to carbon nanotube exposure is a topic for future research.

Investigations of *O. mykiss* exposed to SWNT indicated some lesions in the brains (*42*), an effect that would raise serious concerns about the ecotoxicological effects of these materials if they were indeed determined to be the cause of such effects. Smith and coworkers (*42*) exposed *O. mykiss* to SWNTs (0.1 to 0.5 mg/L) for 10 days and found altered trace metal concentrations, specifically elevated Cu and Zn, in the brain. *O. mykiss* were also more aggressive, had higher ventilation rates, and poorer buoyancy control compared to control fish indicative of alterations in behavior. Similar behavioral changes have been observed as a result of fish exposure to waterborne pollutants which may be linked to underlying alterations in brain functioning (*56*). However, both effects on behavior and

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trace metal homeostasis in trout exposed to SWNTs were also evident in solvent controls and the overall contribution of SWNT to these toxicities is uncertain. Brain pathologies were, however, not related to solvent exposure. Necrotic cell bodies and small foci of vacuoles were evident to varying extents in brains from all SWNT exposed fish, and swelling of blood vessels on the ventral surface of the cerebellum was observed and suggestive of vascular injury in these fish. Enlarged blood vessels could be due to hyperaemia as a result of respiratory distress generated by occlusion of gills by the accumulated SWNTs. Histological changes in the brains of *O. mykiss* were not detected after 6-week dietary SWNT exposure (500 mg/kg food) (*39*) and whether lesions in the brain reported in Smith and coworkers were mediated by toxicities at the gill or absorption of SWNT from the aqueous phase is unclear.

There are, however, some impacts that appear to be nano-size effects or are related to the intrinsic properties of the CNTs. For example, derivitization of the carbon nanotubes to give them various functional groups dramatically impacted their toxicity to *Ceriodaphnia dubia* with positively charged functional groups increasing acute toxicity and hydrophilic functional groups eliminating it (*22*). Carbon nanotubes with specific functional groups that enhance the nanotube's toxicity may indeed cause elevated risks if they are present in water bodies at sufficiently high concentrations, and thus risk assessment for carbon nanotubes should take into consideration the functional groups on the carbon nanotubes. In a separate study, small fluorescent nanocarbon byproducts were shown to increase life-time mortality of estuarine copepod *Amphiascus tenuiremis* at a concentration of 10 mg/L (*46*), while purified SWNTs did not have an effect at this concentration. This nanocarbon fraction had average lengths less than 18 nm and widths and heights near 1 nm and were thus much smaller than the purified SWNTs. Investigating whether similarly small SWNTs would have elevated toxicity is a topic for future research.

One major concern for the environmental relevance of these experiments is the extent to which carbon nanotubes would remain suspended in aquatic ecosystems, or whether the nanotubes would rapidly form aggregates and settle out of the solution. This issue will not be discussed at length in this chapter, but it has been studied extensively (*57–61*), and natural organic matter appears to be one of the primary influential factors (*39*). Additionally, the relatively low CNT concentrations tested for the water-only exposures are still orders of magnitude larger than those average concentrations estimated to be found in the water phase by modeling (*62*). Lastly, most studies use sonication to suspend the carbon nanotubes in solution, but it is unclear to what extent carbon nanotubes would be similarly well dispersed in ecological systems and whether this process overestimate the capacity for carbon nanotubes to remain suspended in the natural environment thereby potentially overestimating their likely risks.

Investigations of the Ecotoxicity of C_{60} Fullerenes

The toxicity of C_{60} has been investigated in ecotoxicity tests and results have been reported in the literature, which provide some initial information (e.g., LC_{50}

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values) to consider for environmental risk assessments. However, there have been significant technical limitations within the emerging discipline of nanotoxicology, and toxicity of C_{60} reported in previous studies must be critically evaluated to determine if any conclusions can be drawn regarding the toxicity of this NP. The objective of this review is to critically examine some of the previous ecotoxicity research and to assess the weight of evidence for a nano-size effect attributable to C_{60} exposure. Although we are aware of the literature emerging on effects of NPs in microorganisms, this review will focus on studies that have investigated multi-cellular organisms rather than unicellular organisms

Natural production of C_{60} has occurred on earth for as long as combustion of carbon proceeded in the absence of oxygen and evidence indicates that forest fires, volcanic eruptions, and meteoritic impacts can all generate C_{60} (*63*). The issue for environmental nanoscience is whether anthropogenic production of C_{60} will generate significant additional quantities of C_{60}, and if this C_{60} will be released in a manner that will generate excessive exposure in biological receptors to cause negative biological effects. Currently, use of C_{60} in consumer products is limited to a few personal care products (e.g. skin creams, see http://www.nanotechproject.org/inventories/consumer/) and estimates of annual releases of fullerenes to various environmental compartments for the US and Europe are available (*62*). Future applications of C_{60} that will increase releases of C_{60} into the environment should be considered; however, the types of products that use C_{60} will inform on routes of disposal and some routes of disposal are not likely to increase environmental concentrations of C_{60} appreciably. For example, use of C_{60} in consumer products will likely lead to disposal through wastewater or as solid waste, and ultimate removal by incineration or burial in landfills-both projected to lead to very little release of C_{60} into the environment (*62*). Some release of C_{60} into surface waters could occur in effluents from wastewater treatment plants or perhaps if C_{60} is used in the future for environmental remediation projects, and, in either case, understanding the ecotoxicity of C_{60} in soils or in surface waters will be important.

Issues Regarding Toxicity of C_{60}

Numerous articles have hypothesized that toxicity of C_{60} is a consequence of oxidative stress (e.g., review (*19*)) and this hypothesis is consistent with the ability of C_{60} to generate ROS under specific conditions (*64*, *65*). Generation of ROS is clearly a consequence of the nanoscale characteristics of this NP, and, if oxidative stress occurs in organisms exposed to C_{60}, then this could be considered evidence of a nano-size effect attributable to this NP. However, the ability of C_{60} to generate ROS has been reported to be limited to when the NP exists as an individual fullerene (i.e., C_{60} dissolved within a solvent) rather than within an aggregate of nC_{60} in the aqueous phase (*66*). If C_{60} does generate ROS when organisms are exposed, oxidative stress can be expected with consequent effects on biological processes.

A central question regarding the toxicology of C_{60} is whether uptake and distribution of the NP is required for toxicity to occur or whether toxic effects (e.g., ROS) can be exerted without transport of C_{60} across epithelial membranes.

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Evidence for uptake of C_{60} across cell membranes is limited, and the most convincing cases have been investigations of pulmonary toxicity in which rodent models have been exposed to nC_{60}. Macrophages were found to contain C_{60} after exposure and appeared to be involved in clearance of the NPs from alveolar surfaces. When nC_{60} has been injected (intraperitoneal (ip)) into rats, transport to tissues was reported and accumulation appeared in the liver, kidney, and spleen occurred as would be expected from the acute (up to 1000 mg/kg) doses administered (*67*). Uptake after oral administration in rats indicated that water soluble $^{14}C_{60}$ (generated by preparation of C_{60} in saline containing 0.2 % Tween 80) was minimal and that the administered C_{60} was voided with feces (*68*). Other oral exposures of rodent models to C_{60} have reported no indications of toxicity in the exposed organisms (*67*, *69*)). No information on dermal uptake of C_{60} is available, although one study reported no skin irritation in humans exposed for 96 h with a skin patch (*70*). The accumulation of C_{60} on the surface of tissues within (e.g., on alveolar surfaces, (*71*)) or on the external surfaces has been documented in various organisms (e.g., (*72*)), but the toxicological consequences of this accumulation are uncertain. Fullerenes did not appear to be readily absorbed based on microscopic examination of microvilli by TEM and most fullerenes were present as large aggregates within the gut lumen of *Daphnia magna* (*73*). Additionally, fullerene accumulations within the gut lumen appeared to be limited by the size of the gut rather than the aqueous phase concentration, again suggesting minimal systemic absorption into the organism. It is possible that accumulation of nC_{60} could influence respiratory processes in some organisms or alter digestive system function during dietary exposure without uptake of the NPs across epithelial membranes; however, whether this would constitute a nano-size effect is questionable. Within the ecotoxicity literature there is no clear evidence of uptake of C_{60} across epithelial membranes.

Ecotoxicity of C_{60} in Aquatic Organisms

Challenges of testing toxicity of nanomaterials include careful characterizations of numerous particle-related properties (discussed in detail in (*2*)) of starting materials and accurate determination of physicochemical properties during exposure (*19*). The ability to obtain C_{60} of relatively high purity (e.g., >99.9 %) that can be generated without use of toxic catalysts (e.g., metals used in generation of CNTs, (*74*)) combined with considerable previous research on properties of C_{60} (*75*) provide a strong foundation for toxicity studies. However, the extreme insolubility of C_{60} ($<10^{-9}$ mg/L) (*76*) and tendency to form colloidal aggregates of nC_{60} (*77*) that have a strong affinity to ad/absorb substances (*78*) from the aqueous media generate scenarios that make testing toxicity difficult and limit comparability among studies. While numerous review articles demand that careful characterization of C_{60} physicochemistry be conducted during toxicity tests (e.g., (*19*)), there is not a consensus on what would constitute sufficient characterization during exposures and no reports have to our knowledge related any physicochemical property of nC_{60} to toxicity. Due to the complexity and changing physicochemistry of nC_{60} that is inherent in environmentally relevant exposures, complete understanding of nC_{60} behavior

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may be an unrealistic goal, and such an undertaking may not even be necessary if these properties are not shown to dramatically impact any toxic effects observed after organisms are exposed to fullerenes.

Generation of nC_{60} in the aqueous phase has been conducted by several techniques and each technique has limitations regarding environmental relevance and implications on toxicity assessment. C_{60} is soluble in organic solvents (*79*), and the solvent tetrahydrofuran (THF) has been found to be particularly useful to produce relatively consistently sized nC_{60} after transfer into the aqueous phase and removal of THF by evaporation (*80*). However, the configuration of nC_{60} enables retention of THF (and other solvents e.g., toluene) within C_{60} aggregates that confounds subsequent evaluations of physicochemistry and toxicity (*81*). nC_{60} can also be generated by addition of C_{60} to pure water and ultrasonication over varying periods of time; however, transmission of high energy on the nanoscale to nC_{60} can change surface chemistry and perhaps generate functionalized fullerenes with different properties (*82*). Long-term (weeks to months) stirring of C_{60} in water (both in natural light and in dark) can lead to the formation of nC_{60} in a manner considered by some to be most environmentally relevant (*72*); however, the extent of formation of hydroxylated C_{60} on the surface of nC_{60} over time is unknown as are the consequences of such changes on environmental fate and ecotoxicity.

The hypothesis that C_{60} (THF generated nC_{60}) can induce oxidative injury in aquatic organisms was supported in early studies (*83–85*) but has subsequently been refuted as techniques for investigating toxicity of C_{60} have been refined. Toxicity attributed to C_{60} in those studies is more likely linked to THF decomposition products as demonstrated in a study with zebrafish (*86*) and further confirmed in subsequent research (*87*). Results that nC_{60} (THF generated nC_{60}) does not generate oxidative injury (or any other toxic effects) when THF and THF decomposition products are removed (*88*) convincingly rejected the hypothesis that C_{60} was responsible for the toxicity reported in studies that have used THF-nC_{60}. Despite this evidence, numerous articles continue to cite studies that have used THF-C_{60} to indicate toxicity of C_{60} (e.g., (*89*)). Results of THF-nC_{60} investigations demonstrate the challenges of testing the toxicity of NPs, but are not acceptable for further discussion about the toxicity of nC_{60} (*88*).

Oxidative stress has been reported in fish exposed to nC_{60} generated by techniques other than solvent exchange and could appear to support the hypothesis that C_{60} can generate ROS and cause toxicity. In fathead minnow *Pimephales promelas*, significant induction of CYP2-like isozymes and elevated lipid peroxidation (liver, gill, brain) was reported (although data was not shown) after 48-h exposure to water stirred nC_{60} (*90*). Results from some of the same investigators as Zhu et al., (*90*) report subsequently that there was no effect of water stirred nC_{60} on CYP2-like isozymes in *P. promelas* or the Japanese medaka *Oryzias latipes* or evidence of lipid peroxidation, which led to the conclusion that traditional biomarkers of oxidative stress were not adequate to demonstrate effects of C_{60} (*91*). Chronic (32 day) exposure to water-stirred nC_{60} had subtle but significant decrease in growth of carp *Crassius auratus* and some significant changes in antioxidant enzyme activity (catalase, superoxide dismutase) in some tissues, but effects, although statistically significant, were not related to

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concentration of nC_{60} (0.04, 0.2, 1.0 mg/L) (*92*). Shinohara et al. (*93*) examined the potential for oxidative injury in common carp *Cyprinus carpio* brains to result from nC_{60} exposure and demonstrated that changes in indicators of oxidative stress were actually a consequence of the assay technique when nC_{60} is present and that if the assay was conducted under lighted conditions then oxidative stress was detected. These results could explain the inconsistencies in oxidative stress indicators reported in the study of Zhu et al., (*92*). In female *Fundulus heteroclitus*, glutathione levels were variable but significantly elevated after exposure to 2.5 and 10 mg/L water-stirred nC_{60}, but no other toxic effects were detected (*72*). Six-week dietary exposure to 500 mg C_{60}/kg food, in juvenile rainbow trout *Oncorhynchus mykiss*, did not cause any changes in oxidative stress endpoints in all major body systems considered (*39*). Overall, the link between ROS-related toxicity and exposure to C_{60} is questionable and has not been separated adequately from effects of vehicle solvents or assay techniques.

The toxicity of C_{60} has been investigated in various aquatic invertebrates and there is evidence of negative consequences of exposure. Investigations that have used nC_{60} generated by THF will not be considered further here for the reasons indicated above; however, nC_{60} produced by long-term stirring in water can affect some aquatic invertebrates. Filter feeding invertebrates can accumulate nC_{60} within their digestive tract and also nC_{60} have been described to adhere to organism surfaces (e.g., *Daphnia magna*, (*94*). Results of acute toxicity in *D. magna* indicate a lack of a dose response to nC_{60} (*85*, *95*) and inability to achieve 100 % mortality even at concentrations up to 500 mg/L (*83*). Of interest are sub-acute responses including reductions in growth (decreased molting) and reproduction in *D. magna* (*95*), and indications that the accumulation of nC_{60} within the digestive tract and on body surfaces may have caused physical disruption and perhaps limited uptake of nutrients. In eastern oysters *Crassostrea virginica* exposed to nC_{60}, toxicity was reported in development of embryo and larval forms (*96*); however, the nC_{60} was prepared by solvent exchange (solvent was toluene) and the contribution of the solvent on toxicity was not completely determined. Within the C_{60} ecotoxicity literature in invertebrates there is evidence for physical effects consequential to the accumulation of nC_{60} aggregates on tissue surfaces; however, there is no evidence for toxicity by other mechanisms (e.g., oxidative stress etc.). Physical disruption of tissue surfaces is a reasonable consequence of accumulation of nC_{60}, but does not constitute a "nano" effect. Unfortunately, controls for a particle effect (e.g., inclusion of amorphous carbon black as a treatment) have not been conducted to determine if effects of surface accumulation of nC_{60} are unique to C_{60} or a general organism response.

Ecotoxicity of C_{60} in Soils and Sediments

In the sole study of fullerene toxicity to multi-cellular soil organisms, no effects were observed on earthworm mortality, reproduction, or growth at food concentrations up to 1000 mg C_{60} per kg food (*31*). There are not yet any published studies on the ecotoxicity of fullerenes in sediment dwelling organisms. While the current results with earthworms suggest minimal C_{60} toxicity to organisms in soils,

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additional research is needed to more fully evaluate the potential risks of fullerenes in these ecosystems.

Conclusions

As the emerging nanotechnology industry matures there is an important need for guidance on the development of this technology to appropriately consider the risks posed by the intentional and unintentional release of NPs into the environment. Nanoparticles do have unique properties, and there are, therefore, risks of novel toxic effects; however, the precautionary principle must be balanced by critical evaluation of the evidence obtained from investigations of toxicity of NPs. Early speculation regarding the potential for ecotoxicity of C_{60} and CNTs was prudently based on understandings of the properties of these NPs. Now that numerous investigations on the toxicity of C_{60} and CNTs have been completed, it is appropriate to re-visit the early speculation and determine how well it is supported by experimental evidence. Evidence for a nano-size effect attributable to C_{60} has not been demonstrated when confounding factors of the experimental design and assay techniques (e.g,, vehicle solvents etc.) are controlled in ecotoxicity studies. Likewise investigations with CNTs have not consistently supported a nano-size related effect, although nano-size toxic effects may have been implicated in a small number of studies. A particular limitation in the connection between C_{60} or CNTs and toxicity in multicellular organisms is that uptake of these NPs across epithelial membranes through normal exposure routes (integument, respiratory surfaces, gastrointestinal tract) is extremely low. Toxic effects exerted on tissue surfaces have been documented but either did not include appropriate controls (e.g., amorphous carbon black) or controls indicated similar effects to NP treatments suggesting that a nano-size effect was unlikely. Not detectable, or extremely low, absorption of C_{60} and CNTs across epithelial membranes and accumulation within tissues (i.e., not accumulation within gut lumen or attached to tissue surfaces) indicates that biomagnification through the food web is not a likely scenario for these NPs after release into the environment. This review is by no means the final word on this topic as techniques for measuring toxicity of NPs and most appropriate effect endpoints to consider are likely to continue to evolve; however, continued discussion of C_{60} and CNT ecotoxicity should move forward from the evidence based on existing ecotoxicity data rather than on early speculation of potential novel toxicity from these NPs. Testing for nano-size effects should continue, but based on existing evidence, nano-size related ecotoxicological effects should not be expected for these NPs.

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