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Deposition of carbon nanotubes by a marine suspension feeder revealed by chemical and isotopic tracers

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HIGHLIGHTS

- CNTs decrease the filtration rate of mussels by as much as 24%.
- Metals in CNTs and their $\delta^{13}C$ can be used to quantify CNTs in biological samples.
- Mussels exposed to CNTs deposit high concentrations of them in biodeposits.
- CNTs accumulate mainly in gut tissue of mussels during exposure.

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ABSTRACT

Carbon nanotubes (CNTs) are one of the few truly novel nanomaterials and are being incorporated into a wide range of products, which will lead to environmental release and potential ecological impacts. We examined the toxicity of CNTs to marine mussels and the effect of mussels on CNT fate and transport by exposing mussels to 1, 2, or 3 mg CNTs l^{-1} for four weeks and measuring mussel clearance rate, shell growth, and CNT accumulation in tissues and deposition in biodeposits. We used metal impurities and carbon stable isotope ratios of the CNTs as tracers of CNT accumulation. Mussels decreased clearance rate of phytoplankton by 24% compared with control animals when exposed to CNTs. However, mussel growth rate was unaffected by CNT concentrations up to 3 mg l^{-1}. Based on metal concentrations and carbon stable isotope values, mussels deposited most CNTs in biodeposits, which contained >110 mg CNTs g^{-1} dry weight, and accumulated about 1 mg CNTs g^{-1} dry weight of tissue. We conclude that extremely high concentrations of CNTs are needed to illicit a toxic response in mussels but the ability of mussels to concentrate and deposit CNTs in feces and pseudofeces may impact infaunal organisms living in and around mussel beds.

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1. Introduction

Carbon nanotubes (CNTs) are cylindrical fullerenes made from either graphite or carbon-containing gas. Most nanomaterials share similarities to their larger counterparts but engineered CNTs are novel carbonaceous structures, making them one of the few examples of a new engineered nanomaterial [1]. CNTs are also similar in shape and size to asbestos, raising speculation that they may be carcinogenic [2]. Despite being potentially very hazardous to human health, CNTs are produced and released into the environment in significant quantities, mainly in propane and natural gas emissions [3,4]. Relatively little consideration has been given to the impact that CNTs may have on the natural environment. Here we focus on the potential impact of CNTs on marine mussels, an ecologically and economically important organism in coastal marine ecosystems.

Several factors common to other engineered nanomaterials influence the toxicity of CNTs. They carry heavy metal impurities that may be toxic [5,6], and can generate reactive oxygen species (ROS) in biological cells that cause injury or disrupt cellular functions [7]. CNTs with relatively long aspect ratios may be more toxic to organisms than smaller CNTs [8,9], leading to injuries associated with apoptosis in macrophage cells and the disruption of immune system function [10]. *In vivo* experiments revealed that CNTs accumulate on external surfaces of *Daphnia magna* bodies impeding movement [11], and can accumulate in their guts interfering with feeding [12]. CNTs can hinder respiration of rainbow trout (*Oncorhynchus mykiss*) by irritating or damaging gill tissues [13]. In only one study, however, did researchers attempt to

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quantify CNTs in or on the organism: Petersen et al. [12] found that *D. magna* accumulated up to 6% of their dry mass in CNTs. Other studies have relied on microscopy and spectroscopy to qualitatively identify the presence of CNTs in or on organisms. Relying on labor-intensive direct visual observations to quantity CNT exposure, uptake, accumulation, and depuration of CNTs limits our capacity to identify and predict the impact of CNTs on organisms and the ecosystems they inhabit.

Here we test the potential impact of CNTs in marine coastal ecosystems by exposing the marine mussel, *Mytilus galloprovincialis*, to CNTs and determining toxicity, biodeposition, and accumulation of these synthetic nanomaterials. We chose mussels as our model species because of (1) their abundance in coastal ecosystems where pollutants may be in relatively high concentrations and (2) their status as representative suspension feeders, making them vulnerable to nanomaterial impacts [14]. We exposed mussels to CNTs for four weeks and measured their clearance rates and growth, and quantified CNT biodeposition and accumulation. The majority of studies conducted so far have detected the presence of unlabeled CNTs by light and electron microscopy [3–5], near-infrared imaging [15,16], and Raman spectroscopy [17]. Methods are being developed to quantify CNTs in water using Raman spectroscopy [18]. Other studies have employed radiolabeled CNTs [12,19]; however, modifying CNTs with radioisotopes or other labels may alter how the CNTs interact with organisms and their environment and requires special instrumentation not often found in toxicology labs [20]. To circumvent these issues, we used $\delta^{13}C$ stable isotope ratios and metal impurities associated with CNTs as tracers to evaluate CNT biodeposition by mussels, *via* feces and pseudofeces, as well as accumulation in mussel tissue. Based on impacts seen in other organisms [11,13], we hypothesized that CNT exposure would impact clearance, respiration, and growth rates of mussels. Mussels are able to reject undesirable particles by encapsulating them in a mucous matrix as pseudofeces that are expelled before entering the gut. Therefore, we predicted that this mechanism would decrease mussel exposure and that mussels exposed to CNTs would (1) decrease clearance rate, (2) decrease growth rate, (3) deposit CNTs in feces and pseudofeces, and (4) accumulate CNTs in tissues. We tested these predictions using linear regression models that describe the dose and time dependence of clearance rate as well as deposition and accumulation of CNTs.

2. Experimental

We purchased mussels from Taylor Shellfish Farms (Seattle, WA, USA) and kept them in flowing, sand-filtered seawater for one week prior to the start of experiments to allow for recovery after shipping. Mussels spawned immediately after addition to acclimation tanks. Spawning was completed after 1–2 days at which point dead mussels were removed from tanks. No food was provided during this acclimation period. We chose mussels for the experiment based on total shell length (TL), which we measured using digital calipers (0.01 mm) along the longest axis of the mussel.

2.1. CNT preparation

We purchased purified, electric arc-discharge single wall carbon nanotubes (CNTs) from Carbon Solutions, Inc. (Riverside, CA). The CNTs had a tube diameter ranging from 1.22 to 1.96 nm, aggregated to 644 ± 206 nm in purified water (Barnstead Nanopure, Thermo Fisher Scientific, Waltham, MA, USA, resistivity > 18 MΩ cm), had a length of 100–1000 μm, and contained trace amounts of Y and Ni [21,22]. We prepared 1 g l^{-1} stock suspensions of CNTs prior to each water change by adding CNTs to purified water containing 500 mg l^{-1} of Suwannee River natural organic matter (International Humic Substances Society, St. Paul, MN, USA). We sonicated this suspension in a bath sonicator for 15 min, then diluted this suspension to 100 mg l^{-1} using 0.45 μm filtered seawater and sonicated again for 15 min. We measured size of CNTs in this suspension immediately after sonication using dynamic light scattering (Nano ZS90, Malvern Instruments, Ltd, Worcestershire, UK); mean diameter in seawater was 3660 ± 200 nm. We then added the appropriate amount of this suspension to polyethylene cups containing 0.45 μm filtered seawater and 100 μl of feed to obtain concentrations of 1, 2, or 3 mg l^{-1} and a final volume of 150 ml.

2.2. Toxicity of CNTs

We determined toxicity of CNTs in four week exposure experiments. We placed 80 mussels, measuring 30 ± 1 mm, individually into aerated polyethylene cups containing 150 ml of 0.45 μm filtered seawater and kept them at 14 °C. CNT exposure treatments were 0 (control), 1, 2, and 3 mg CNTs l^{-1}, which were added with 100 μl of phytoplankton feed (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) to attain a phytoplankton concentration of 1.3×10^6 cells ml^{-1}. These CNT concentrations were chosen to ensure that mussels would not remain closed during the experiment and to provide enough material to enable us to quantify CNTs in our samples. Aeration was set so that phytoplankton did not settle to the bottom of the container. We changed the water-CNT mixture every other day and added 100 μl of feed to each container. Water potentially containing CNTs was collected and disposed of as hazardous waste.

We measured clearance rate weekly of five mussels in each group by removing 1 ml of water from each container immediately after feeding and every 20 min thereafter for 2 h. We estimated phytoplankton concentration of the samples by measuring *in vivo* chlorophyll fluorescence using a fluorometer (Model 7200-043, Turner Designs, Sunnyvale, CA, USA). A standard curve of the relationship between phytoplankton concentration and fluorescence was established by manually counting a serially diluted set of the phytoplankton feed and determining fluorescence of these samples. Clearance rate was calculated for each individual by fitting an exponential function to the decrease in phytoplankton concentration over time as follows:

$$y(t) = a \times e^{kt} \quad (1)$$

where $y(t)$ is the phytoplankton concentration at time, t, a is the intercept, and k is the rate of decline in phytoplankton concentration. The same mussels were used throughout the experiment for clearance rate measurements.

We measured growth rate weekly of three mussels in each group. Mussels were collected from cups, TL was measured as above, and mussels were frozen for tissue analysis. Daily growth rate was determined by calculating the change in an individual's TL and dividing by the number of days the mussels had been exposed to CNTs.

2.3. Biodeposition and accumulation of CNTs

We sampled three mussels and their feces and pseudofeces weekly from each treatment group to measure accumulation and biodeposition of CNTs. We measured TL and froze mussels immediately after collection. Later, we thawed the mussels and dissected and separated gill, mantle, and viscera from the remaining tissue, rinsing these tissues in purified water twice. To collect biodeposits we poured out the overlying water in each container, collected the remainder into a vial, allowed the biodeposits to settle overnight, poured off the supernatant and gently rinsed the biodeposits with purified water three times, allowing them to settle overnight between rinses. Because these biodeposits are held together in a

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