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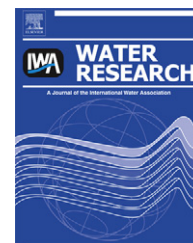
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Stability, metal leaching, photoactivity and toxicity in freshwater systems of commercial single wall carbon nanotubes

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ABSTRACT

Carbon nanotubes (CNTs) are exciting new materials that have been intensively researched and are becoming increasingly used in consumer products. With rapid growth in production and use of CNTs in many applications, there is the potential for emissions to the environment and thus research is needed to assess the risks associated with CNTs in the environment. Here we show that commercial CNTs differ in their stability, photoactivity, metal leachate, and toxicity to freshwater algae. The behavior between raw and purified variants of the CNTs differs considerably; for example purified CNTs are generally more photoactive, producing singlet oxygen and superoxide, while raw CNTs show little or no photoactivity. Residual metal catalysts differ based on synthesis method used to prepare CNTs and thus may be comprised of elements with varying degrees of toxic potential. Influenced by pH and other constituents of the natural waters, our work shows that metals can leach out from all the commercial CNTs studied, even purified versions, albeit at different levels in many natural waters. As much as 10% of the total residual nickel leached from a purified CNT after 72 h. Aqueous concentrations of molybdenum leached from a different purified CNT were nearly 0.060 mg L⁻¹ after 72 h. With little sample preparation, CNTs are dispersible in most freshwaters and stable for several days. Not all tested CNTs were toxic; for those CNTs that did induce toxicity we show that photoactivity, not metal leaching, contributes to the toxicity of commercial CNTs to freshwater algae, with growth rates significantly reduced by as much as 200%.

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1. Introduction

Single wall carbon nanotubes (CNTs) are rolled sheets of sp²-bonded carbon, valued for their unique electronic and physical properties. CNTs are easily functionalized with both covalent and non-covalent moieties and thus have

uses in biomedical applications, sensors, electronics and consumer products. The various functionalizations and dispersal methods have been shown to influence CNT toxicity, which can be enhanced or decreased, as well as their fate (Kennedy et al., 2009). Considering the myriad current and potential uses of CNTs and the intensity of

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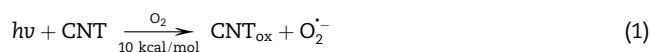
research and application development, CNTs are likely to enter the environment during several stages in their life cycle (Templeton et al., 2006). Although detection of CNTs in real environmental samples is technically difficult (Petersen et al., 2011), predicted environmental CNT concentrations in surface waters are less than $1 \mu\text{g L}^{-1}$. However, CNT concentrations could be as high as $23 \mu\text{g L}^{-1}$ at sewage treatment plant outflows (Gottschalk et al., 2009; Mueller and Nowack, 2008). Interestingly, much work has focused on the research and development of applications of CNTs, but only a few studies have focused on the behavior and risks associated with CNTs in environmentally relevant conditions (Petersen et al., 2011; Hutchison, 2008; Wang et al., 2008; Robichaud et al., 2005; Lecoanet et al., 2004). Preliminary ecotoxicity studies have shown that CNTs are toxic to algae, fish, daphnids and other organisms (Schwab et al., 2011; Petersen et al., 2009; Smith et al., 2007). Transport studies showed that humic acid, a common environmental constituent, dispersed CNTs and increased their mobility in the environment (Wang et al., 2008). Similarly, work by Lecoanet et al. (2004) showed that environmental conditions increase the transport of the otherwise hydrophobic CNTs (Lecoanet et al., 2004). While a preliminary risk analysis found a low risk for CNTs in soils and sediments, CNTs in the aqueous phase were more toxic (Petersen et al., 2011; Robichaud et al., 2005).

Work with single and multi-walled carbon nanotubes indicates that CNT stability may be explained by Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Yi and Chen, 2011; Chen et al., 2010). As such, the stability of CNTs in natural waters is influenced by solution chemistry, with the degree of stability important for predicting the risks of environmental release (Chen et al., 2010). CNTs are hydrophobic, due to strong Van der Waals forces along their length axis, and readily aggregate in water and other organic solvents (Wang et al., 2008; Dhimi et al., 2008). However, several studies have shown that the attractive pi–pi interactions between CNTs can be influenced by various surfactants (Lecoanet et al., 2004; Chen et al., 2010; Islam et al., 2003). Natural organic matter (NOM), a ubiquitous component in natural waters, is a natural surfactant that may stabilize CNTs under many conditions (Wang et al., 2008). If these natural water constituents are able to produce stable dispersions of CNTs, then CNTs will likely be mobile in the environment and interactions with aquatic organisms will be more probable. Conversely, natural environments with low NOM availability and/or high ionic strength may result in unstable CNT dispersions that result in CNT sedimenting out, limiting transport, reactivity and exposure to pelagic organisms (Yi and Chen, 2011).

As a consequence of material synthesis, metal residues are contained within most CNT matrices (Li et al., 2004; Alvarez et al., 2008; Nikolaev et al., 1999). Depending on the CNT application, the CNTs may be treated with acid to reduce the residual metals (Hirsch, 2002). While purified CNTs may contain only a small percentage of the metal catalysts, raw CNTs can contain as much as 40% metal by weight. To date little work has been done to quantify the release of metal residues from CNT matrices in natural waters, or the toxicological implications of the bioavailable

metals (Schwab et al., 2011; Hull et al., 2009; Blaise et al., 2008). It was previously thought that CNT purification removed almost all metals that could leach in solution, but there may be some residual metals since metal dissolution is kinetically limited and most purification processes are too short in duration (Liu et al., 2008). In addition, acid purification degrades the protective CNT encapsulation of the metals (Liu et al., 2008; Shen et al., 2005), and also exposes defects in the CNT structure. We hypothesize that leached metals may be a factor in CNT toxicity, and that purer CNTs will thus be less toxic to aquatic organisms.

In addition to metal leaching, the photoactivity of CNTs and most nanomaterials in natural waters is poorly understood. Several nanomaterials are known to be photoactive, with the ability to produce reactive oxygen species (ROS) (Pickering and Wiesner, 2005; Brunet et al., 2009; Badireddy et al., 2007; Miller et al., 2012; Bennett and Keller, 2011). Few studies have investigated the interactions of CNTs with natural light, and fewer have done so under environmentally relevant conditions (Miller et al., 2012). Alvarez et al. (2008) showed that surfactant dispersed CNTs in water were hydroxylated after irradiation with UV light, indicating that CNTs reach a photoinduced excited state that reacts with adsorbed water to produce a hydroxylated CNT surface (Alvarez et al., 2008). Photohydroxylation of CNTs is a light-mediated introduction of CNT defects, which are known to increase reactivity of the CNTs (Alvarez et al., 2008; Hirsch, 2002). It has also been shown that semiconducting CNTs have a longer photoinduced excited state than metallic (conducting) CNTs and are thus more reactive (Alvarez et al., 2008). In waters designed to mimic environmentally relevant conditions, the interactions of CNTs with sunlight have been generally characterized as photoreaction and phototransformation (Chen and Jafvert, 2010). Researchers working with artificial dye–CNT complexes have shown that a photoactivated dye can donate an electron to CNTs and subsequently this electron can be donated to adsorbed O_2 , producing the reactive superoxide anion (O_2^-) (Martinez and Galano, 2010). Chen and Jafvert (2010) have shown that, in the presence of sunlight, carboxylated–CNTs were able to produce singlet oxygen ($^1\text{O}_2$) and the much more reactive hydroxyl radical (OH^\bullet) and $\text{O}_2^{\bullet-}$ (Chen and Jafvert, 2010). The production of ROS by CNTs is likely to follow Type I photocatalytic, Type II photosensitized, or dye sensitized photochemical pathways (Chen and Jafvert, 2010; Joshi et al., 2008; Cho, 2002):



where $h\nu$ is light, ${}^3\text{CNT}$ is excited triplet-state carbon nanotube, CNT_{ox} is an oxidized carbon nanotube, $\text{NOM} - \text{CNT}$ is an electronically coupled complex, NOM^\bullet is the NOM radical, NOM_{ox} is an oxidized NOM molecule and CNT^\bullet is a CNT radical. The reaction in Eq. (1) proceeds when adsorbed dissolved O_2 extracts an electron from the photoactivated CNT

(Joshi et al., 2008); the reaction in Eq. (2) proceeds when a CNT is excited to the triplet-state and then transfers the energy to adsorbed ground state O_2 (Joshi et al., 2008); and the reaction in Eq. (3) occurs when the dye molecule (NOM) is excited and donates an electron to the adsorbed CNT. The CNT then conveys the electron to adsorbed dissolved oxygen, and finally superoxide is produced. CNTs have been shown to be excellent electron acceptors that can effectively transport electrons (Baskaran et al., 2005). Considering the efficacy of the photoactive dye-CNT complex, it is then reasonable that a natural analogue could be photoactive as well. NOM is an effective light harvester and has been shown to participate in ligand to metal charge transfer reactions (Cho, 2002).

CNTs have been shown to induce toxicity via a few mechanisms, one of which requires adsorption of a cell to the CNT and subsequent mechanical piercing of the cell by the CNT (Kang et al., 2007). Wang et al. (2010) showed that degree of dispersability influences toxicity and may be mediated by improving the interaction of the CNT with cell membranes or by increased bioavailability. The ability of dye-CNT complexes to induce toxicity to microbes was shown to be mediated by the photoactivated production of ROS (Banerjee et al., 2010). Leaching of the residual metal catalyst associated with CNTs can influence organismal performance (Blaise et al., 2008). However, Schwab et al. (2011) reported no effect of residual metals and that shading by the CNTs accounted for the observed reduced growth rates (Schwab et al., 2011). In fact, Schwab et al. (2011) showed that algae and CNTs form irreversible aggregates, ostensibly due to hydrogen binding between the CNTs and the algal surface, which reduces the photosynthetic capability of the algae (Schwab et al., 2011).

Our objective in this study was to determine the stability, photoactivity and toxicity of commercial CNTs in natural waters. Due to the potential importance of residual catalyst metals, we selected CNTs representing purified and raw (i.e. as synthesized) forms to measure extent and kinetics of metal leaching. To assess the potential toxicity from CNT photoactivity and metal leaching we exposed planktonic freshwater algae, which are at the base of the food chain and thus can serve to better assess the environmental implications. The results provide the information needed to perform a more comprehensive assessment of the potential impacts of a release of commercial CNTs into freshwater systems.

2. Materials and methods

2.1. Carbon nanotubes

Five different commercial CNTs, representing three different common manufacturing processes, were used as received. High-pressure carbon monoxide (HiPCo) CNTs (NanoIntegris, IL, USA) were received in raw and purified form, purified via thermal treatment, denoted HR and HP in the rest of this document. Electric arc-discharge CNTs from Carbon Solutions (CA, USA) were also received in raw and purified form, purified via air oxidation, and are identified as P2-R and P2-P. An additional purified CNT, SG65-P, prepared using a cobalt and molybdenum catalyst (CoMoCat) was received at relatively high purity, purified via HF dissolution of residual catalyst, from Southwest Nano Technologies (OK, USA). CNT purity, as well as other physicochemical data of the CNTs, are presented in Table 1.

2.2. Natural waters

Hyung and coworkers previously showed that environmental samples of NOM can effectively stabilize multi-walled carbon nanotubes in purified water, and thus we also used NOM in our studies (Hyung et al., 2007). Suwanee River natural organic matter isolate (International Humic Substances Society, St. Paul, MN) was dissolved in Nanopure water and stirred for 24 h to achieve a stock solution of 500 mg L^{-1} ; the stock solution was added to deionized water (DI) to produce 10 mg L^{-1} NOM water. Groundwater was obtained from a shallow agricultural monitoring well (2 m) in Santa Clara, CA. Stormwater was collected from a storm drain destined for a lagoon at UC Santa Barbara. Seawater was taken directly from the Pacific Ocean (Santa Barbara, CA), autoclaved and filtered through $0.22 \mu\text{m}$. Freshwater growth media (denominated Synthetic Freshwater) was made in accordance with EPA method 1003.0 and also contained 1 mg L^{-1} NOM, as a result of CNT stock dispersion. Due to potential interference with the metal leaching experiments, EDTA was not added to the EPA growth media. Table 2 presents the key characteristics of the natural waters, with a more detailed characterization in Keller et al. (2010). The pH was measured using an Oakton pH meter (Ion 510 series, Fisher Scientific). Conductivity was measured with a Fisher Scientific Traceable Conductivity, Resistivity, and TDS Meter (Fisher Scientific). Calcium and magnesium were

Table 1 – Physicochemical characterization of the five tested single-walled carbon nanotubes. UV–Vis: absorbance spectrometer; TEM: transmission electron microscopy; TGA: thermogravimetry; ICP-MS: inductively coupled plasma mass spectrometer; EPM: electrophoretic mobility.

Properties	Technique	Unit	P2-P	P2-R	HP	HR	SG65-P
Tube length	TEM	nm	450–2000	100–1000	100–1000	1000–5000	500–1500
Tube diameter	UV–vis	nm	1.22–1.96	1.22–1.96	0.70–1.13	0.70–1.13	0.68–0.98
Bundle diameter	TEM	nm	7.5 ± 2.4	6.3 ± 2.0	13 ± 3.5	10.7 ± 3.6	7.4 ± 2.3
SWNT purity	TGA	wt. %	84.6	64.8	76.8	63	78.7
Metal impurity	ICP-MS	wt. %	0.55 ± 1.60	11.91 ± 32.71	16.01	31.35	0.65 ± 4.73
			Y + Ni	Y + Ni	Fe	Fe	Co + Mo
Zeta Potential in H_2O	Zeta PALS	mV	-29.3 ± 2.43	-25.4 ± 0.7	-39.9 ± 2.7	-22.3 ± 0.7	-23.4 ± 1.0
EPM in H_2O	Zeta PALS	$10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$	-2.21 ± 0.18	-1.91 ± 0.06	-3.00 ± 0.21	-1.67 ± 0.05	-1.76 ± 0.08

Table 2 – Characteristics of the natural waters used in these experiments. Analytical techniques detailed in Supporting information. TOC = total organic carbon.

Natural water	pH	TOC (mg L ⁻¹)	Conductivity (mV)	Ca ²⁺ (mg L ⁻¹)	Mg ²⁺ (mg L ⁻¹)
Stormwater	7.1	822.5	424	46.78	13.03
Groundwater	7.9	745.6	217	447.10	224.90
DI + 10 mg L ⁻¹ NOM	6.5	499.2	9.07	<1	<1
Synthetic freshwater + 1 mg L ⁻¹ NOM	6.8	60.5	92.9	1.20	2.90
Seawater	8.1	19.2	2.58 × 10 ⁴	398.00	1361.40
DI	6.6	0.0	1.43	<1	<1

measured using inductively coupled plasma-atomic emission spectroscopy (Thermo iCap 6300 Model).

2.3. Stability experiments

Static and shaken stability tests were conducted. The effect of shaking on CNT stability was evaluated to test the influence of shaker tables used for the toxicity experiments. Dry CNT powder was weighed and bath sonicated to prepare stock suspensions of 100 mg L⁻¹ CNTs in either 1 mg L⁻¹ or 10 mg L⁻¹ NOM. The CNT stock suspensions were sonicated for 10 min, stirred for 10 min and then sonicated once more for 10 min (Branson 2510, USA). Aliquots of the CNT stock suspensions were dispensed directly into the natural or synthetic waters and then sonicated for 10 min to prepare the experimental samples. Using a 1 cm quartz cell (Suprasil quartz, Fisher Scientific, USA), CNT stability was monitored via UV/Vis absorbance at 550 nm (Shimadzu Biospec 1601, Japan) following (Schwyzer et al., 2011). Based on previous studies, NOM does not absorb strongly at 550 nm and assuming the absorbances of NOM and CNTs are additive, UV-vis spectroscopy provides a tractable method for measuring CNT concentration (Wang et al., 2008; Schwyzer et al., 2011). Suspended CNT concentration was determined by constructing a calibration curve (Supplementary Information, Fig. SI-1) of CNTs dispersed in DI + 10 mg L⁻¹ NOM ([CNT] = 1, 10 and 20 mg L⁻¹) and then measured via UV/Vis spectroscopy at 550 nm. The standards were sonicated for 30 min. Stability experiments conducted with freshwater media were performed in control suspensions without algal cells, to avoid interferences. Stability was monitored for the 72 h exposure period. CNT hydrodynamic diameter was measured by dynamic light scattering at 24 and 72 h (Zetasizer ZS-90, Malvern Instruments, Worcester-shire, UK).

2.4. Metal leaching experiments

To prepare the CNT dispersions for metal leaching experiments, 0.5 mg of a given CNT was weighed and added to 50 ml of each of the water matrices. The solutions were sonicated for 10 min and then placed in a temperature-controlled room at 20 °C. Five ml samples of the supernatant were withdrawn daily, filtered at 0.2 µm (sterile surfactant free cellulose acetate syringe filters, Thermo Scientific, USA), centrifuged at 10,000 RPM for 10 min and then 4 ml of supernatant were withdrawn for analysis on an inductively coupled plasma-

atomic emission spectrometer (ICP-AES, ThermoCahn iCap 6300, USA). Standards to calibrate the ICP-AES were obtained from High Purity Standards (South Carolina, USA).

2.5. Photoactivity experiments

Photoactivity was probed with electroparamagnetic resonance spectroscopy (EPR) (EMX Plus EPR Spectrometer, Bruker, USA) and UV-vis spectroscopy (Shimadzu UV3600 UV-Vis-NIR Spectrometer, Japan). We followed well established EPR spin-trapping techniques using 2,2,6,6-tetramethyl-4-piperidinol (TEMPL, Sigma Aldrich, USA) to test for ¹O₂ production, and 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Caymen Chemical, Florida, USA) to test for OH[•] (Mothilal et al., 2004; Zhao et al., 2008). To test the singlet oxygen assay, rose bengal (Fisher Scientific, USA) was used at 10 mM in deionized control experiments as a positive confirmation of ¹O₂ detection (Mothilal et al., 2004). Beta-carotene (Fisher Scientific, USA) is a quencher specific to singlet oxygen and was used to provide evidence that an observed signal is in fact from singlet oxygen (Sachindra et al., 2007). The following EPR parameters were used: microwave power, 10 mW; modulation amplitude, 4.0 G; modulation frequency, 100.0 kHz and at least 3 scans were averaged to yield each EPR spectrum. The experimental solution containing 100 µl of 100 µM TEMPL was added to 800 µl of DI water and 100 µl of a CNT dispersed with 10 mg L⁻¹ NOM. The quartz cell (4 mm Precision Suprasil EPR Sample Tube, Norell Inc, USA) containing the mixed solution was placed directly into the EPR cavity and was irradiated via a broadband (ca. 200–2500 nm) Xe arc lamp (300 W m⁻²). The EPR intensity was monitored over time and the double integral of the absorbance was used to determine ROS production. Superoxide production assays were also conducted with the Xe arc lamp and was monitored on the UV/Vis spectrometer using 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT, MP Bio-medicals, USA) following (Brunet et al., 2009). One ml of 1000 µM XTT was added to 9 ml of 10 mg L⁻¹ CNTs dispersed in 10 mg L⁻¹ NOM. A stir bar was added and the sample was exposed to light. Samples were withdrawn periodically, filtered with a 0.22 µm nitro-cellulose syringe filters (Millipore, USA) and analyzed. The absorbance at 470 nm was used to measure the reduction of XTT. To confirm that superoxide was indeed produced, 10 KU superoxide dismutase (SOD, MP Biomedicals, USA), a commonly used anti-oxidant selective for superoxide, was added to the XTT/CNT/NOM solution (Brunet et al., 2009).

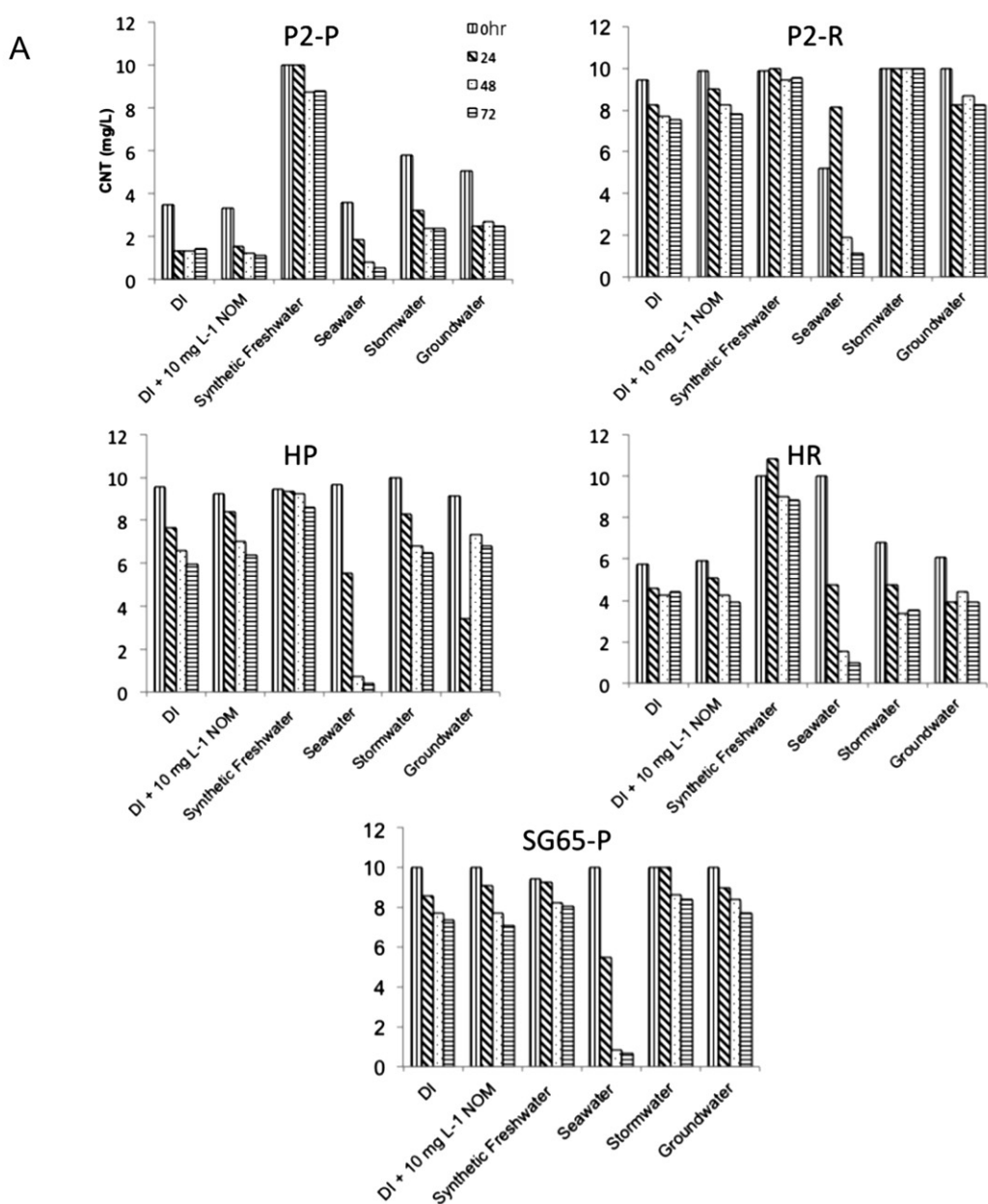


Fig. 1 – Panel A: Dispersability and static stability over 72 h for five commercial CNTs in various natural waters. Panel B: Hydrodynamic diameter of five commercial CNTs in natural waters at 24 and 72 h. The concentration of NOM in DI + NOM and synthetic freshwater were 10 and 1 mg L⁻¹, respectively.

2.6. Freshwater toxicity assay

Pseudokirchneriella subcapitata were obtained from Ward Scientific (NY, USA) and maintained in freshwater growth media (EPA method 1003.0). Two hundred ml flasks were inoculated with 10 mg L^{-1} CNT and ca. $10,000 \text{ cells ml}^{-1}$. Ten ml of the CNT stock suspensions were added to the synthetic freshwater media resulting in final CNT concentrations of 10 mg L^{-1} . Experiments contained NOM concentration of 1 mg L^{-1} , by starting with the CNT stock dispersed in 10 mg L^{-1} NOM. The algae were incubated in a temperature-controlled room (20°C) and kept under fluorescent lighting (14 h light, 10 h dark, intensity ranged from 100 to $120 \mu\text{mol m}^{-2} \text{ s}^{-1}$) on a shaker table (New Brunswick Scientific Co., NJ, USA). UV-fluorescent lights were used to provide lighting in the short wavelength region of the spectrum (295–365 nm, UVA-340, Q-Lab Corp., Cleveland OH), characteristic of the near-surface natural waters (4.5 W m^{-2}) (Tedetti and Sempéré, 2006). Two experimental lighting conditions were used in this work, UV and no UV. UVA intensity in the UV treatment averaged 4.5 (S.E. 0.1 , $n = 6$) W m^{-2} and UVB averaged 4.1 (S.E. 0.2 , $n = 6$) W m^{-2} . The removal of the UV in the no UV treatment was accomplished using UV-filtering acrylic (Plexiglas G UF-3, Ridout Plastics, USA) that blocked 98% of the UV. The UV intensity was measured with a broadband radiometer Model

UVX, UVP Inc. Upland CA). Cell density was measured as *in vivo* chlorophyll via fluorescence at 0, 24, 48 and 72 h (Trilogy, Turner Designs, USA).

Metal exposure experiments were conducted in the same manner as described above for the CNTs. Metal salts were dissolved to produce 1 g L^{-1} stock solutions that were diluted into the synthetic freshwater to generate solutions at 0.01, 0.1, 1 and 10 mg L^{-1} metals. Metal salts were obtained from Sigma Aldrich (USA).

2.7. Statistical analysis

Statistical significance was determined using the Student's *t*-test in Microsoft Excel.

3. Results and discussion

3.1. Dispersability and stability

Much work has focused on the stability of CNTs, both for material applications and due to environmental implications; however, little work has focused on a critical condition that directly influences the amount of CNTs that may be suspended in aqueous media, dispersability. Dispersability refers

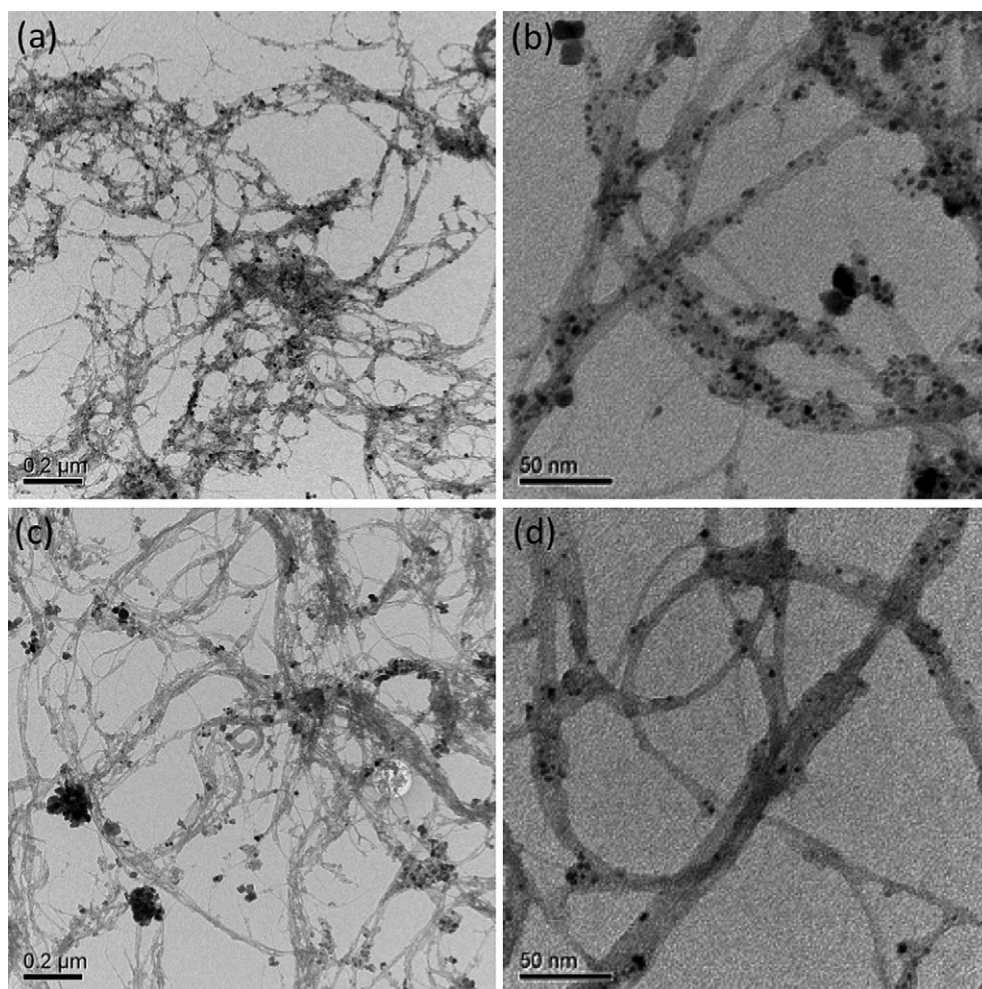


Fig. 2 – TEM micrographs of (a, b) HR and (c, d) HP CNTs show metal particles within.

to the ability of media constituents to disperse CNTs, i.e. reduce aggregation. In the case of natural waters, dispersability results from the interplay between NOM and other organics that are able to disperse CNTs and ionic constituents and net DLVO interactions that drive aggregation. The dispersability of a given natural water can then influence the amount of CNTs available in the water column and when considered with stability provides a more comprehensive measure of the exposure risks in a particular environment. Operationally we define dispersability as the initial concentration (time 0) in a stability study (Fig. 1, Panel A) for a specific water matrix. The observed initial dispersability indicates that waters high in organic matter (i.e. stormwater, groundwater and DI-NOM) will result in higher dispersed fraction of a CNT loading. Three of the commercial CNTs dispersed well initially in seawater. The lowest stability was observed in seawater, for all 5 CNTs, even after adding 10 mg L⁻¹ alginate, a natural seaweed based dispersant (data not shown). Although most of the CNTs dispersed well in the high organic matter media, P2-P had low dispersability in almost all waters, with rapid aggregation and settling. Differences in dispersibility and stability in different waters may also have to do with the nature of the NOM present in different water matrices.

Due to the non-covalent binding of organic matter onto CNTs and the subsequent introduction of electrosteric repulsion and a degree of hydrophilicity from the functional groups on the organic matter, we hypothesized that waters with the highest organic content and lowest conductivity, would yield the most stable dispersions (Stormwater > Groundwater > DI-NOM > Freshwater > Seawater). As can be seen in Fig. 1, natural waters with high concentrations of organic matter were generally most effective at producing stable dispersions in these static experiments, although stability in the same medium varied widely for the different CNTs. For example, P2-R and HP were essentially 100% stable after 3 days in stormwater, while the other CNTs had lower stability in the same water matrix. This implies that CNT stability is critically linked with NOM availability and that for waters low in NOM organisms in the water column are most at risk in the short-term, while in the longer term benthic organisms are more likely to be exposed. Because most natural waters have NOM concentrations between 1 and 10 mg L⁻¹ (Thurman, 1985), algae are then susceptible to CNT exposure. The stability data reported here show that depending on CNT type and environmental conditions, the fate of a given CNT will likely be quite different. SG65-P may have unintended functionalization from the acidic purification, which may explain its high dispersibility. Although DI-NOM initially (first few hours) produced stable dispersions of all CNTs, as observed by (Wang et al., 2008; Hyung et al., 2007) stability decreased significantly for some CNTs over 3 days. In seawater, the CNTs were nearly absent from the water column, due to lower organic content and higher ionic strength. In general shaking had little influence on CNT stability, compared to the static stability studies (Fig. SI-2).

As predicted by DLVO, after a short time large CNT aggregates (several microns) formed in high ionic strength waters and smaller aggregates (less than 1 micron) were observed in low ionic strength waters. CNT aggregate size increased significantly over the experimental period in the high ionic

strength waters, contributing to the rapid settling observed in seawater (Fig. 1 Panel B, see Table SI-1 for standard error). Marginal size changes were observed over the three-day experimental period for CNTs in waters with higher NOM, synthetic freshwater with 1 mg L⁻¹ NOM, stormwater and groundwater. Although the hydrodynamic diameters measured here do not directly reflect the exact size of nanotubes, the DLS measurement does provide semi-quantitative information about the dispersion state and hydrodynamic diameter of CNTs (Smith et al., 2009). This is confirmed by a recent study indicating that DLS is a valuable technique for estimating the dispersion state of multi-walled carbon nanotubes (Wang et al., 2010).

3.2. Metal leaching

The five selected CNTs were synthesized by various methods and therefore contain different catalysts in varying amounts, Ni and Y for P2-P and P2-R; Fe for H-P and H-R; and Co and Mo for SG65-P (Table 1). Representative TEM micrographs of both HP and HR show that although metals are lower in the purified form, large metal particles can be present in the purified samples (Fig. 2) (Chiang et al., 2001). Although some CNTs are purified via acid washing and other methods, the residual metal

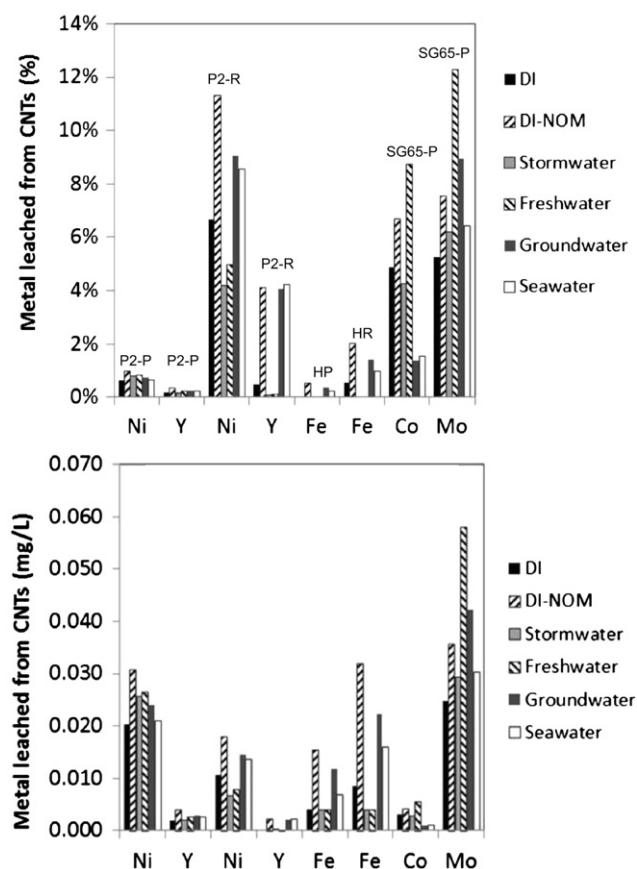


Fig. 3 – Metals released after 72 h from five commercial CNTs in deionized water (DI), DI + 10 mg L⁻¹ NOM, stormwater, synthetic freshwater, groundwater, and seawater, as (top) percent of the initial metal and (bottom) metal concentration in suspension.

catalyst in the dry powder can amount to as much as 2.15% of the P2-P, 5.38% of the SG65-P and 16.01% of the HiPCO CNTs.

The dissolved metal concentrations that result after dispersion of 10 mg L⁻¹ CNT in various waters is on the order of 1–60 µg L⁻¹ (Fig. 3) after 72 h, but this represents leaching of up to 12% of the residual metal, particularly for Ni, Co and Mo. Surprisingly, the highest metal concentrations observed were not from the two raw materials. While P2-R did leach more total metals than P2-P, HR and HP leached comparable amounts of Fe. It is also important to note that SG65-P leached more metal than any of the other CNTs. As a percentage, the purified materials leached more of the metals contained within their CNT matrices. There was some variability in the total amount leached in different waters, with a higher level in DI + 10 mg L⁻¹ NOM and freshwater for most CNTs, which correspond to natural waters with lower pH. Most of the leaching occurred during the first 24 h, with the concentrations remaining constant after that point. It is possible that the concentrations reached the solubility limit within a short time, since most of the metals are sparingly soluble.

3.3. Photoactivity

For all purified CNTs, the reduced form of the XTT probe was observed after ca. 10 min, which indicates the production of

O₂⁻ (Fig. 4, Left Panel). Increased exposure time correlated well with the reduction of XTT. The raw forms of the CNTs (P2-R and HR) did not reduce XTT to any measurable extent and thus likely did not produce O₂⁻. The addition of SOD decreased the formation of XTT, which provides more evidence for the production of superoxide (data not shown). Work by Chen and Jafvert (2010) showed that at the same CNT concentration used in our studies, irradiated CNT-COOH was able to increase the absorbance of XTT from 0 to 0.08 after nearly 40 h; in the present studies P2-P and H-P were able to do so after ~90 min. The differences in photoactivity, between our work and that by Chen and Jafvert, are possibly due to experimental conditions, e.g., lighting treatment, but may also arise from differences in photoactivity due to the nature of the CNTs used in this study.

Production of ¹O₂ was observed after only 20 min of irradiation for two purified materials, P2-P and SG65-P, and also from P2-R (Fig. 4, Right Panel). After a 30 min light exposure, a small signal was observed for HP but not HR. Because the CNTs were dispersed with a known photosensitizer (NOM) capable of independently producing ¹O₂, we conducted controlled experiments with NOM in the absence of CNT, but did not observe any appreciable production of ¹O₂ at even higher concentrations of NOM (10 mg L⁻¹, Fig. SI-3). We also conducted the CNT photoactivity assay in the presence of

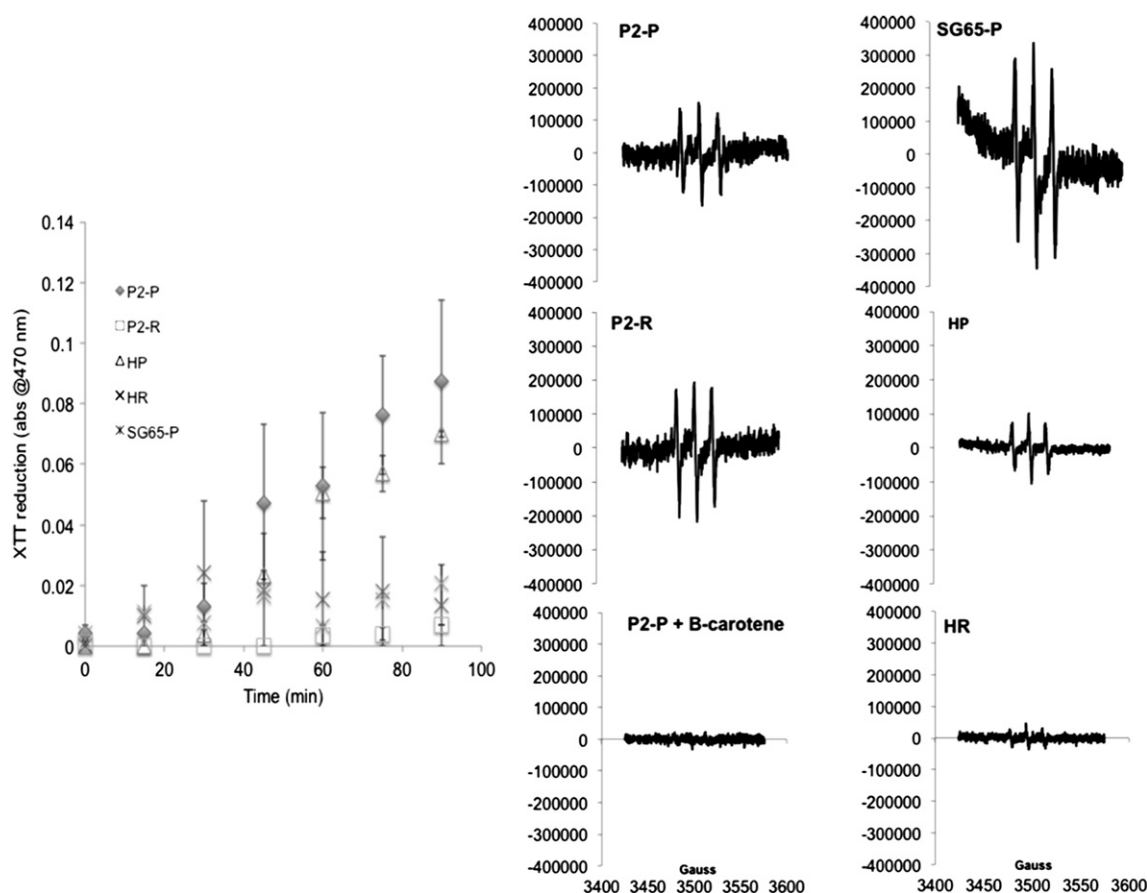


Fig. 4 – Panel A: Production of O₂⁻ by the three purified CNTs, P2-P, HP and SG65-P when irradiated with broadband light, as indicated by the ability to reduce XTT; and Panel B; EPR spectra characteristic for ¹O₂ for P2-P, P2-R, SG65-P when irradiated for 20 min and a marginal signal from HP when irradiated with light for 30 min. The addition of beta-carotene, a ¹O₂ scavenger, effectively quenches the production of ¹O₂ by P2-P.

beta-carotene, a known scavenger of $^1\text{O}_2$, and photoactivity was not observed, providing more evidence that $^1\text{O}_2$ was formed (Fig. 4, Right Panel).

With the exception of HR, all CNTs were photoactive, producing superoxide and/or singlet oxygen, with P2-P and HP best able to produce O_2^- while SG65-P, P2-P and P2-R were most effective at producing $^1\text{O}_2$. The basis of the observed photoactivity differences between pure and raw CNTs is outside the scope of this study, but the observations may be due to the scavenging ability of the amorphous carbon associated with raw samples (Dunne et al., 1992). We also tested for OH^\cdot generation, but did not observe any production of OH^\cdot (data not shown).

3.4. Toxicity

Our toxicity experiments had two lighting treatment levels, UV and no UV, designed to correspond to marked differences in optical transitions (photoactivity) for the CNTs (Fig. 5). All results are compared to controls with no CNTs under the same conditions. The growth rate of *P. subcapitata* was decreased, with statistical significance (statistical analysis presented in Table SI-1), by all P2-R and P2-P treatments, HP without UV, and by SG65-P with UV, (Fig. 5, Panel A). The HR CNTs were not

toxic in any treatment. SG65-P CNTs were the most toxic, with reduced growth rates for both treatments and a negative growth rate in the UV treatment. There was no trend observed for toxicity differences between pure or raw CNTs, with both pure and raw CNTs significantly toxic in 50% of their respective treatments.

3.5. Phototoxicity

Our studies indicate a limited role of CNT mediated photoactivity on the observed reduced algal growth rates. All CNTs have strong absorption bands in the UV and thus may be able to generate ROS via energy transfer reactions (Fig. SI-4). Additionally, photons in the UV band contain enough energy to make electron withdrawal by O_2 energetically feasible (Joshi et al., 2008), i.e. UV photons at 350 nm contain 82 kcal mol^{-1} and only $7.68\text{--}13.37 \text{ kcal mol}^{-1}$ is required for electron withdrawal from CNTs with similar diameter (Chen and Jafvert, 2010; Joshi et al., 2008). There was an influence of the lighting treatment on growth rates, with the UV treatment and CNTs generally yielding decreased growth rates compared to the corresponding controls (Fig. 5, Panel A). However, the observed differences between the UV and no UV treatments were only reflected in statistically significant reduced growth

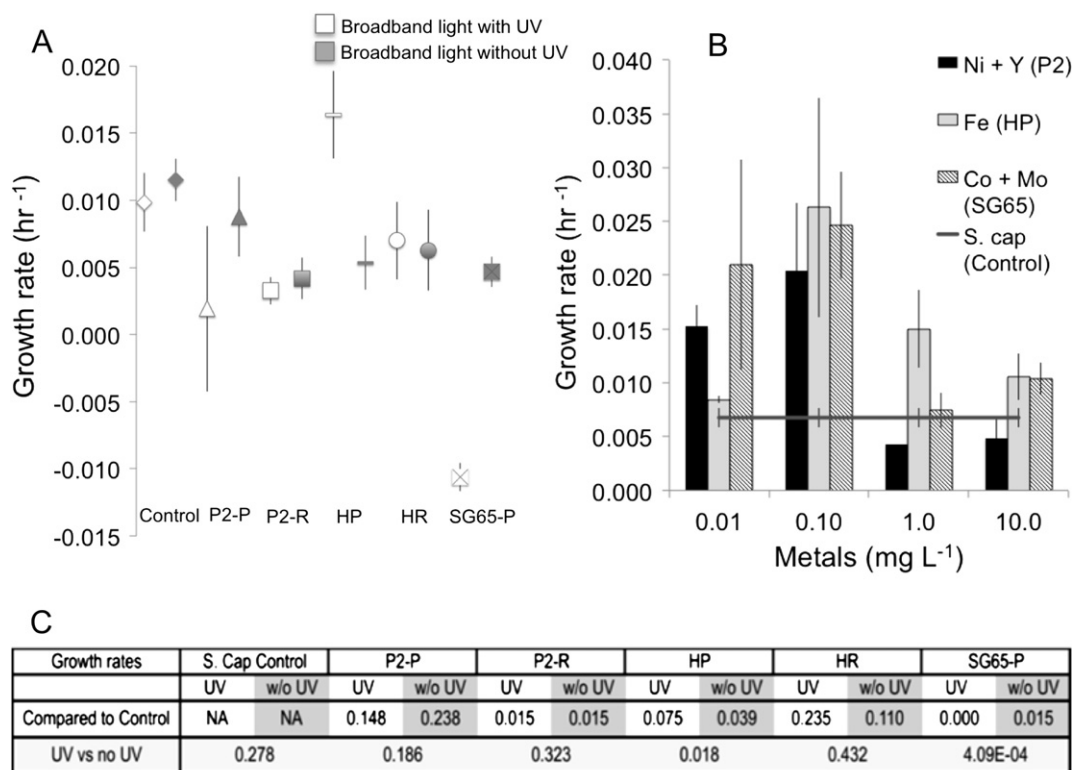


Fig. 5 – Panel A: Reflects growth curves for *P. subcapitata* in the absence and presence of the five tested CNTs with and without UV light. Exposure to 10 mg L^{-1} CNT does negatively influence the growth of algae across most treatments ($n \geq 3$). Each CNT is represented by a different symbol, where the open symbols reflect exposures with UV and the darkened symbols show data for exposures without UV. **Panel B:** Reflects experiments in which *P. subcapitata* were exposed to dissolved metals at various concentrations. The control (i.e. no CNTs) growth rate is 0.0067 h^{-1} , as indicated by the horizontal line. **Panel C:** P-values for statistical analysis (t-test) of the growth rates with the same treatment. The ‘UV vs no UV’ series reflects the statistical significance for the UV and no UV treatment for the same CNT.

rates for SG65-P (p -value 4.09×10^{-4}). There was no statistically significant difference for the control treatment growth for the UV exposure levels, suggesting that UV light was not responsible for reduced growth rates.

3.6. Metals toxicity

After 72 h CNT exposure in the synthetic freshwater the leached metal concentrations (all in mg L^{-1}) for P2-P were 0.027 Ni and 0.003 Y, for P2P2-R were 0.020 Ni and 0.008 Y, for HP and HR were 0.004 Fe and for SG65-P were 0.006 Co and 0.058 Mo. We conducted metal exposure experiments with 0.01, 0.1, 1 and 10 mg L^{-1} for the different metal combinations. At metal concentrations of 0.01 and 0.1 mg L^{-1} the algae were able to grow as well as, or better than, the algae grown in the absence of metals, indicating that metals leaching is likely not the cause of the reduced growth rates in the exposure of CNTs to algae (Fig. 5, Panel B). It is important to note that although NOM has been shown to chelate metals and thus may influence metals toxicity to algae, NOM chelation did not influence the observed toxicity in our experiments (Calace and Petronio, 2004). Our metals dissolution studies were conducted in the absence of NOM and we used those concentration data, representing metals concentrations in the absence of a chelator, for the control metal-algae exposures. We found that metals did not account for the observed reduced growth rates under our experimental conditions. It is important to note that more toxic metals, e.g., Cu, may be present in other CNTs and may in fact induce toxicity, as observed by Hull et al. (2009).

3.7. Shading

The photosynthetic active radiation (PAR), light from 400 to 700 nm, used in this study was $100\text{--}120 \mu\text{E m}^{-2} \text{ s}^{-1}$. We investigated the ability of the CNT suspensions to block PAR and found that at $[\text{CNT}] = 10 \text{ mg L}^{-1}$, P2-P, P2-R, HP and HR reduced PAR by about 10% while SG65-P reduced PAR by ca. 25% (Fig. 6). A photograph of the five CNTs dispersed in synthetic freshwater also shows that the suspensions are optically clear (Fig. SI-5). Work by Mayer and coworkers found that

at similar PAR intensities the growth rate of *P. subcapitata*, the same algae used in our work, was not significantly reduced by even a 50% reduction in PAR from 100 to $50 \mu\text{E m}^{-2} \text{ s}^{-1}$ (Mayer et al., 1998). Thus, we don't believe shading had a significant influence on the observed reduced growth rates in our work.

4. Conclusions

We have shown that CNT dispersability and stability are strongly dependent on the natural waters in which CNTs may be released. As hypothesized, the presence of NOM generally resulted in more dispersible and stable suspensions, although it appears as though higher concentrations of NOM may reduce stability as in the case of 10 mg L^{-1} NOM. While NOM can serve to initially disperse the CNT suspensions, there is a marked decrease in CNT concentrations in waters with high ionic strength. Since many natural freshwaters have $[\text{NOM}] > 1 \text{ mg L}^{-1}$, it can be expected that under these conditions CNTs will remain stable at least for a few days and are likely to readily transport and interact in biotic and abiotic processes. We also show that metal leaching from the CNTs also varies by natural water with most of the residual metal leached after only a day or two. Metal leaching is higher in lower pH natural waters. Metal leaching is observed not only from raw CNTs, but also from purified materials, with between 5 and 10% metal leaching for P2-P (Ni) and SG65-P (Co) in nearly all tested natural waters. All purified CNTs were shown to produce singlet oxygen and superoxide but not hydroxyl radical. The raw CNTs demonstrated different photoactivity, with P2-R able to produce singlet oxygen but not superoxide and HR was essentially inert. The presence of NOM in solution was not sufficient to generate ROS. The hypothesis that, under our experimental conditions, leached metals would be a factor in CNT toxicity proved false, with no observed toxicity from the dissolved metal ions at the concentrations found to leach in our control experiments. In our studies we showed that metal dissolution or shading were not the cause of toxicity and thus propose that CNT photoactivity is a source of toxicity of CNTs to freshwater algae. In our comprehensive study we showed that CNTs are stable in many natural waters, photoactive and in some cases toxic to freshwater algae, but that all of these processes depend on the type of CNTs and environmental conditions.

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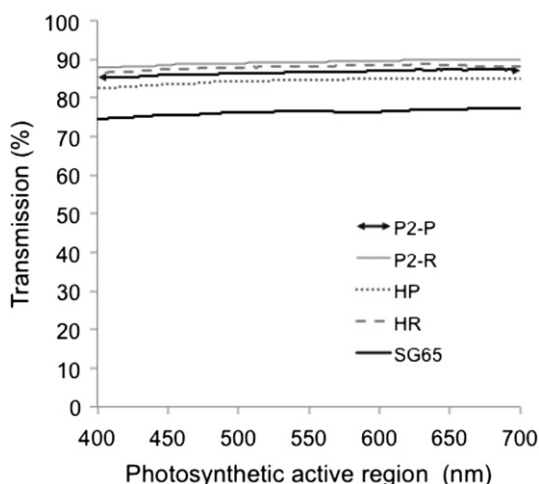


Fig. 6 – Transmission of PAR through CNT suspensions with 10 mg L^{-1} CNT and 1 mg L^{-1} NOM.

Appendix A. Supplementary material

Supplementary material related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2012.12.039>.

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