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Mitigation of the impact of single-walled carbon nanotubes on a freshwater green algae: *Pseudokirchneriella subcapitata*

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Abstract

This study investigates the biological response of *Pseudokirchneriella subcapitata* to single-walled carbon nanotubes (SWNTs) suspended in gum Arabic (GA), using typical 96-hour algal bioassays and long-term growth studies. Changes in algal biomass and cell morphology associated with specific SWNT-treatments were monitored and the mechanisms of observed biological responses investigated through a combination of biochemical and spectroscopic methods. Results from short-term bioassays showed a growth inhibition in culture media containing >0.5 mg SWNT/L and a final GA concentration of 0.023% (v/v). Interestingly, the observed toxicity disappears when GA concentrations are brought to levels ≥ 0.046%. Long-term experiments based on toxic combination of SWNTs and GA showed that *P. subcapitata* would easily recover from an initial growth inhibition effect. Overall, these findings point to the possibility of GA to mitigate the toxicity of SWNTs, making it an ideal surfactant if SWNT suspension in GA does not alter the performance sought from these nanotubes.

Keywords: *Single-walled carbon nanotubes, gum Arabic, Pseudokirchneriella subcapitata, exposure, biological response, mechanisms*

Introduction

Single-walled carbon nanotubes (SWNTs) have attracted the attention of both engineers and scientists because of their anticipated widespread commercial and industrial applications (Baughman et al. 2002; Dresselhaus et al. 2003; Endo et al. 2004). This interest stems primarily from their unique size-related characteristics, such as strength, elasticity, high adsorption capacity, and controllable conductivity, which led to their introduction into a wide variety of commercial products. However, the potential for introduction of SWNTs to the environment during manufacturing stages and during both use and disposal of SWNT-containing products could grow with the economic success of engineered nanomaterials (ENMs). Accordingly, SWNTs entering waste streams would likely interact with ecological functions, and ultimately impact the biosphere. In fact, aquatic systems, such as rivers and lakes, behave as primary environmental sinks by integrating pollutants from atmospheric deposition, terrestrial surface runoffs, and groundwater discharges, raising concerns on the potential implications of SWNTs that might accumulate in these systems (Gao et al. 2009).

Recent research on the biological implications of SWNTs has produced rather conflicting results, in that both toxic and non-toxic effects are reported (Cherukuri et al. 2004; Lam et al. 2004; Lecoanet et al. 2004; Warheit et al. 2004; Jia et al. 2005; Shvedova et al. 2005; Federici et al. 2007; Griffitt et al. 2007; Lin and Xing 2007; Smith et al. 2007; Chou et al. 2008). In addition to studies focusing on the environmental aspects, the anticipated use of SWNTs as biosensors and in medicine (Mattson et al. 2000; Chen et al. 2003; Trommer and Neubert 2005; Liu et al. 2007) has also stimulated research on the investigation of the potential effects of ENMs on humans. In this latter case, preliminary results have raised concerns on the biological effects of SWNTs at both the cell and organism levels (Lam et al. 2004; Warheit et al. 2004; Shvedova et al. 2005; Muller et al. 2006). In fact, the currently observed

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toxic effects of SWNTs have been attributed to several parameters, including the levels and types of metal impurities (Shvedova et al. 2003; Kagan et al. 2006; Koyama et al. 2009), the degree and kind of aggregation in produced SWNTs (Wick et al. 2007; Lin et al. 2009), physical characteristics, such as shape and surface area (Flahaut et al. 2006; Kang et al. 2007, 2008), the type and role of surfactants used to disperse them (Monteiro-Riviere et al. 2005; Sayes et al. 2006; Dong et al. 2008; Simon-Deckers et al. 2008), and more likely a combination of two or more of the above parameters.

The significance of the toxic role of used surfactants stems from the fact that ideal SWNT-suspensions are currently obtained by use of a wide variety of aqueous surfactant solutions, with different chemical properties. Commonly used surfactants include gum Arabic (GA) (Bandyopadhyaya et al. 2002), sodium dodecyl sulfate (SDS) (Vigolo et al. 2000; O'Connell et al. 2002; Moore et al. 2003; Regev et al. 2004; Smith et al. 2007), sodium dodecylbenzene sulfonate (SDBS) (Moore et al. 2003; Attal et al. 2006; Cognet et al. 2007), sodium cholate (SC) (Moore et al. 2003), and Triton X-100 (Islam et al. 2003; Moore et al. 2003; Zhou et al. 2003). Some preliminary studies have also indicated that dissolved natural organic matter could help enhance nanoparticle suspension in aqueous solutions (Hyung et al. 2007). While these surfactants are used to enhance the dispersion/suspension of SWNTs, some can also affect the inherent properties of SWNTs (Garg and Sinnott 1998; Wang et al. 2008; Silvera-Batista et al. 2009), and potentially the interactions between SWNTs and living organisms in toxicity studies (Dong et al. 2008). Our preliminary studies on the effects of surfactant types and SWNTs toxicity on *P. subcapitata* and *Ceriodaphnia dubia* have shown that certain surfactants (e.g., SDBS, SDS, SC, Triton X-15 and Triton X-100) are already toxic to these two model organisms (Gao 2008). Therefore, toxicity studies based on the above model organisms and using SWNTs suspended in such surfactants could not provide an accurate assessment of the effects of pristine SWNTs. In contrast, other surfactants, such as GA (Simon-Deckers et al. 2008; Alpatova et al. 2010) and polyvinyl pyrrolidone, were found to be non-toxic (Gao 2008).

It is then obvious that efforts to reduce or eliminate the risks associated with SWNTs or any other ENMs would require an understanding of the nanoparticle's parameters responsible for toxicity, as well as the mechanisms of interaction between nanoparticle and organism. In this study, a model aquatic organism, the freshwater green algae *P. subcapitata*, also known as *Selenastrum capricornutum* (U.S. Environmental Protection Agency [USEPA] 2002), is used in a series of laboratory experiments to investigate the biological effects of SWNTs under different growth condition scenarios. Quantitative (algal biomass) and qualitative (morphology) changes associated with specific SWNT-treatments were monitored, while the mechanisms of observed biological responses were investigated through a combination of biochemical and spectroscopic methods.

Materials and methods

Preparation and characterization of SWNT suspensions

SWNT suspensions used in this study were prepared by suspending approximately 40 mg of raw SWNTs (Rice HPR 145.1) into 200 mL of an aqueous 1% (m/v) GA solution (Sigma-Aldrich). To facilitate the dispersion process, the mixture was homogenized using a high-shear IKA T-25 Ultra-Turrax mixer for about 1.5 h followed by ultrasonication using a Misonix S3000 for 10 min. The mixture was then ultra-centrifuged at 20,000 rpm (Beckman Coulter Optima L-80 K) for 2.5 h, and the supernatant carefully separated from the aggregated SWNTs at the bottom of the centrifuge tube. The obtained suspensions were then characterized using atomic force microscopy (AFM, Digital Instruments D3100; Veeco Co., Plainview, NY, USA), thermo-gravimetric analysis (TGA, Mettler Toledo TGA/SDTA, Mettler-Toledo International Inc., Columbus, OH, USA), and elemental analysis by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Algal growth in SWNT-containing culture media and SWNT aggregation state in algal suspensions

Pure cultures of *P. subcapitata* were obtained from Hydrosphere Research (Alachua, FL, USA) and cultivated under constant light exposure at room temperature. The algal growth procedure used in this study was adapted from USEPA's *P. subcapitata* 96-h growth inhibition method 1003.0 (USEPA 2002). In dose-exposure studies, 50 mL of a preliminary algal assay procedure (or PAAP) culture medium (USEPA 2002) were transferred into 150 mL Erlenmeyer flasks and sterilized by autoclaving. To investigate the effects of SWNTs on algal growth, the following experiments were conducted.

Effects of exposure to increasing SWNT concentrations in algal cultures with a fixed concentration of GA

In this set of experiments, sterilized culture media were first spiked with aliquot volumes of a concentrated SWNT-suspension to produce growth media with concentration gradients ranging from

0.01–0.5 ppm. For these experiments, the addition of different volumes of the SWNT-suspension to culture media resulted in different final concentrations of GA, which was then corrected by adding needed volumes of a plain GA-solution to obtain uniform final GA concentrations in all growth media. Based on this approach, algal exposure studies were conducted at two different final GA concentrations of 0.023% and 0.046% (v/v). Following the addition of SWNTs and after vigorous mixing, culture media were inoculated with 1 mL of a pure algal suspension (500,000 cells/mL).

Growth experiments were conducted under continuous illumination (~86 $\mu E \cdot m^{-2} s^{-1}$) and both control and SWNT-treated samples were prepared in triplicates, and the different experiments repeated at least twice. Changes in chlorophyll *a* (Chl-*a*) concentrations were used as an indicator of general biological responses and were monitored over time using a Turner Quantech Digital-Filter Fluorometer. Algal growth rates determined from control samples were averaged, assigned a value of 100%, and used as a reference value to report the growth rates determined from SWNT-treated culture media.

Effects of algal exposure to increasing GA concentrations in culture media containing a fixed concentration of SWNTs

The second set of exposure studies used algal culture media with fixed SWNT-concentrations while the final concentrations of GA was varied from 0–0.17% v/v. Experiment conditions and analytical methods were similar to those described in the previous section.

Long-term exposure of algal cells to a pre-identified toxic combination of SWNTs and GA

In addition to the above short-term (96 h) experiments, the pre-identified most toxic combination of SWNT (0.5 ppm) and GA (0.023%) concentrations was used in a long-term (two weeks) exposure study. In this set of experiments, aliquot volumes of SWNTs suspended in GA were added to growing algal suspensions either at the start or at different points along the algal S-growth curve (i.e., additions during lag, exponential, and plateau phases). Algal growth conditions (e.g., culture media, light exposure) and analytical methods used in these long-term experiments were similar to those previously described.

Determination of glutathione as indicator oxidative stress

To investigate the potential mechanisms of observed biological responses, concentrations of reduced glutathione (GSH) were determined using culture media spiked with a fixed amount of SWNTs and increasing GA levels in 96-h growth experiments. The GSH assay was adapted from methods described by Hissin and Hilf (1976) and Cohn and Lyle (1966). Briefly, algal suspensions were first centrifuged at 4°C and 2000 rpm for 15 min. The supernatant was discarded and the pellet was re-suspended in 5 mL of a sodium phosphate buffer (0.1 M sodium phosphate –0.005 M EDTA, pH 8). After 10 min incubation on ice, the mixture was transferred into a vial and sonicated within an ice bath with a Cell Disruptor W-375 (Heat Systems-Ultrasonics INC, Farmingdale, NY, USA). 500 μL of the sonicated suspension were then vigorously mixed with 500 μL of *o*-phthalaldehyde and 4.5 mL of the buffer solution and allowed to react at room temperature for 20 min. The fluorescence of GSH was finally measured with a Turner Quantech Digital-Filter Fluorometer (with emission and excitation wavelengths set at 420 and 350 nm, respectively).

For all of the above algal growth experiments, statistical differences ($\alpha < 0.05$) in observed biological responses between treatments were determined using a single factor analysis of variance (ANOVA).

Determination of the aggregation state of SWNTs in algal culture media

The aggregation state of SWNTs within the culture media at both initial time (t_0) and after 96 h (t_f) was probed by use of the combination of the following techniques. The vis-NIR (near infrared) absorbance and NIR fluorescence spectra of both controls and SWNT-treated algal suspensions were recorded using an ANF-Nanospectralyzer (Houston, TX, USA), with excitation from a 662 nm diode laser. The concentration of SWNTs in all suspensions was determined using Beer-Lambert's Law and the absorbance measured at 763 nm, similar to prior studies (Moore 2005; Parra-Vasquez et al. 2007). Raman spectra were also recorded using a Renishaw Invia Bio Raman with excitation from a 785 nm diode laser.

Transmission electron microscopy (TEM) studies

TEM imaging was conducted at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR), using a Hitachi H-7000 TEM (Hitachi High Technologies America, Inc. Schaumburg, IL, USA). Samples were prepared using methods adopted from the literature (Ellis 2006), and digital images were acquired with a Veleta camera (Veleta – Olympus Soft-Imaging Solutions Corp,

Lakewood, CO, USA) and iTEM software. Briefly, algal cultures were centrifuged at 1300 RPM for 10 min and then fixed with Trumps (1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer), which was from Electron Microscopy Sciences, Hatfield, PA, USA. Fixed cells were stored at 4°C overnight and processed with a Pelco BioWave Microwave (Ted Pella, Redding, CA, USA) to improve fixation. Samples were next washed in 0.1 M sodium cacodylate (pH 7.24), post-fixed with 2% buffered osmium tetroxide, water washed and dehydrated in a graded ethanol series of 25%, 50%, 75%, 95%, 100%, followed by 100% acetone. Dehydrated samples were infiltrated in graded acetone/Spurr's epoxy resin and cured at 60°C. Cured resin blocks were trimmed, cut into thin sections and collected on formvar copper slot grids. Samples were post-stained with 2% aqueous uranyl acetate and Reynold's lead citrate to improve contrast. Sections were then examined with TEM. Digital images were acquired with a Veleta camera (Veleta – Olympus Soft-Imaging Solutions Corp, Lakewood, CO) and iTEM software.

Results

Characterization of SWNT suspensions

An example of collected AFM images and the determined length distribution of the nanotubes is shown in Figure 1. GA-suspended SWNTs exhibited a length distribution ranging from 300–1200 nm. Levels of metal impurities in SWNT-suspensions determined quantitatively by thermo-gravimetric analysis and qualitatively by ICP-AES averaged 13.4% and 17 ppm, respectively.

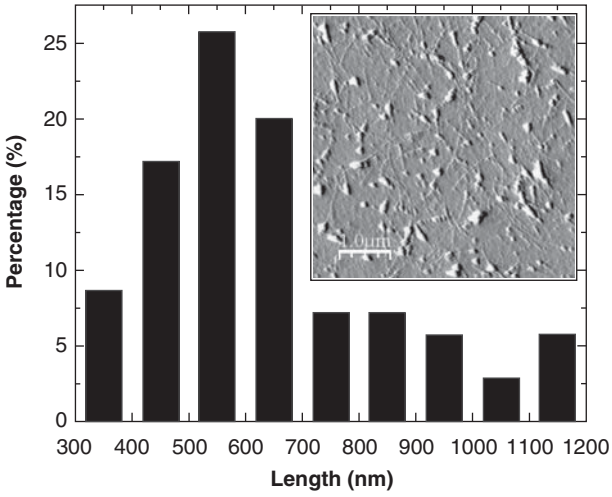

Figure 1. Length distributions of individual single walled carbon nanotubes (SWNTs) suspended in 1% gum Arabic (GA) determined by analysis of AFM image (see inset).

Effect of increasing SWNT concentrations on P. subcapitata growth

Figure 2 shows the growth response of *P. subcapitata* to increasing concentrations of SWNTs in culture media containing fixed GA levels (0.023% and 0.046% v/v) in a series of 96-h growth experiments. The horizontal lines represent the average of algal growth rates determined from control cultures.

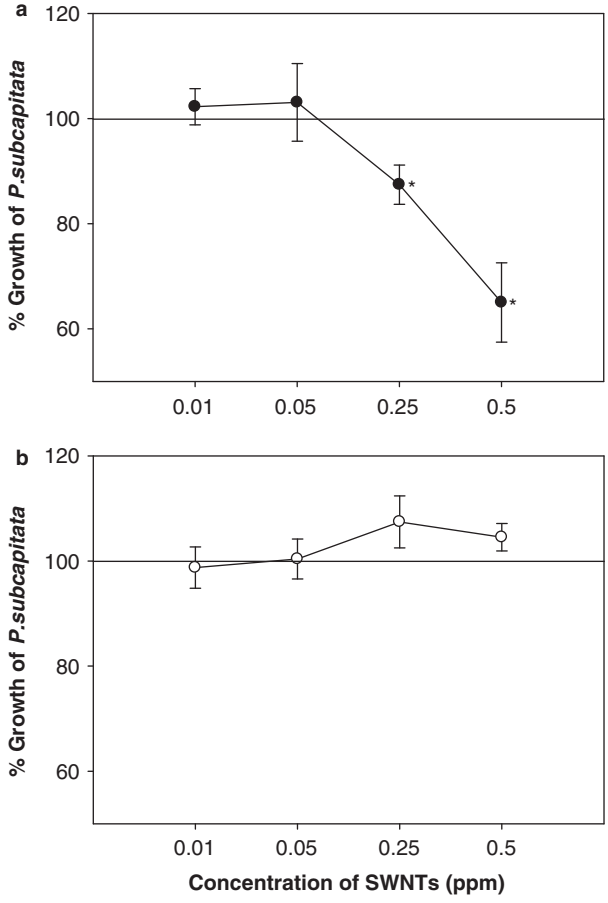

Figure 2. Effect of increasing concentrations of SWNTs on the growth of *P. subcapitata* in a standard 96-h chronic algal assay. The final concentration of gum Arabic (GA) used as surfactant to suspend SWNTs was adjusted in all culture media to a final concentration of 0.023% (*1a*), and 0.046% (*1b*). The horizontal line represents the growth of *P. subcapitata* in control culture media (i.e., culture media containing only GA but no SWNTs). Growth rates obtained from controls were assigned a value of 100% and biological responses measured in treated samples normalized to the control. (*) indicates a significant difference as compared to controls ($\alpha < 0.05$).

SWNT-treated media containing a final GA concentration of 0.023% show no growth inhibition effects for SWNT concentrations ranging from 0.01–0.05 ppm. However, a negative impact on algal growth and, therefore, toxicity is observed for SWNT concentrations >0.05 ppm (Figure 2a). In contrast, when the same SWNT-concentration gradient is used with increased final GA-concentration from 0.023–0.046% v/v, SWNT-toxicity disappears and a tendency for growth bio-stimulation is observed (Figure 2b). Overall, for this tested range of SWNT concentrations, the increase in levels of GA, a non-toxic surfactant for the model organism used here, alters the toxicity of SWNTs. The potential mechanisms are discussed later herein.

Interactions of P. subcapitata with SWNTs introduced in culture media at different points along the growth curve

In this set of experiments, aliquot volumes of SWNT-suspensions were added to algal growth media prior to the inoculation with pure algal suspension (Figure 3a) or subsequently after an initial growth period (Figure 3b, 3c, 3d). These experiments focused on the effect of the above determined toxic combination of 0.5 ppm of SWNTs and 0.023% of GA (Figure 2a) on different growth stages of *P. subcapitata*. The results show that when added prior to inoculation of the culture medium with algal cells, the growth inhibition is pronounced in the first 150 h (% growth decreased below ~60% of growth observed in control samples). However, this inhibition appears to be reversible as the algal growth recovers over time, reaching growth rate levels similar to that of the control after 200 h (Figure 3a).

The addition of SWNTs several hours after the inoculation of culture media with algal cells showed different behaviors. First, SWNT addition to culture media 24 h after the initiation of the algal growth (Figure 3b) had a less inhibitory effect than SWNT introduction during the exponential growth phase (96 h, Figure 3c). For the former (Figure 3b), algal growth rebounds from the initial inhibition following the introduction of SWNTs, and increases to reach levels that exceed growth rates in control samples. In contrast, the addition of SWNTs after 96 h had a pronounced inhibition effect, and although a general trend towards recovery is observed over time, growth rates in these culture media did not return to levels measured in control samples (Figure 3c). Finally, the addition of SWNTs at the end of the exponential growth phase or plateau (Figure 3d) leads to a drop in growth without recovery due to the limited nutrients in these batch cultures. Overall, in these long-term

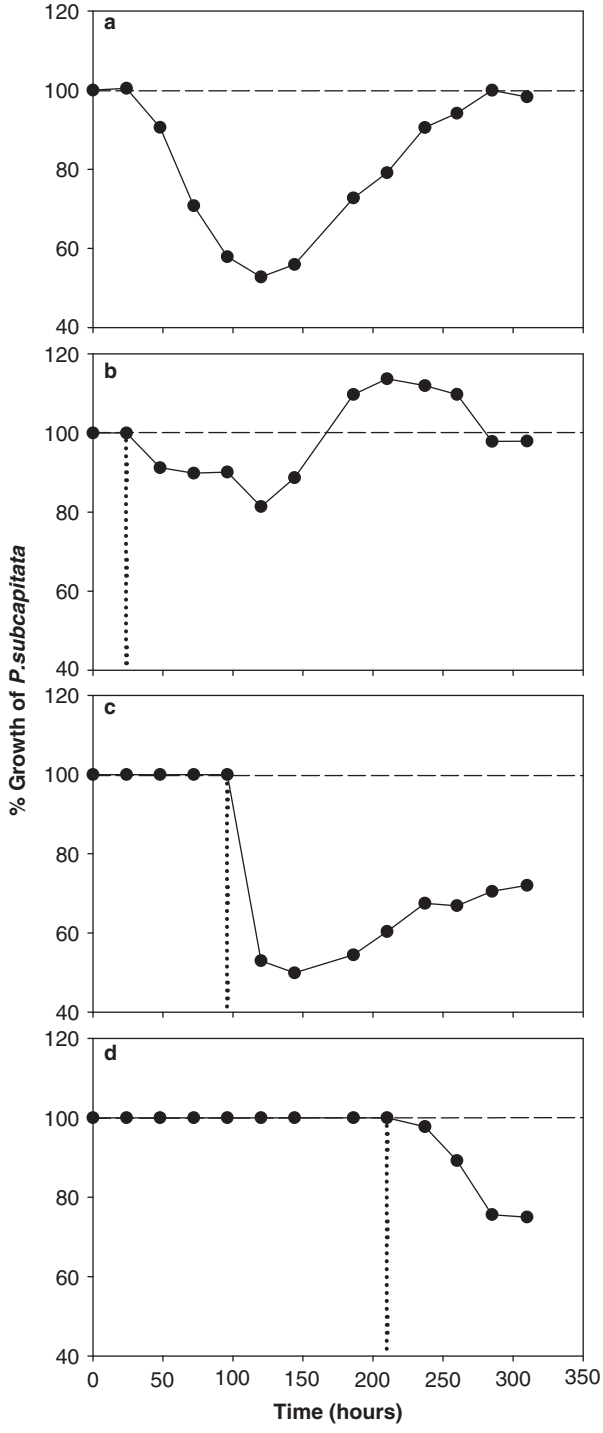

Figure 3. Biological response of *P. subcapitata* to SWNT introduction to the culture medium at different algal growth phases. (a) SWNTs added prior to inoculating with the algal cells, i.e. time t_0; (b) SWNT addition after 24 h of growth; (c) SWNT addition after 96 h and during the exponential growth phase, and (d) SWNT addition after 216 h of growth (near plateau). The horizontal lines represent the percentage of algal growth in control culture media (i.e., culture media containing only GA but no SWNTs), which have been assigned values of 100%, and the observed effects in treated samples are normalized to the control. Vertical dotted lines indicate the point of SWNT addition to the culture media.

growth experiments, the tested toxic mixture of GA-SWNT would delay algal growth, but would not result in a complete growth inhibition of *P. subcapitata*. Our preliminary experiments using SWNTs suspended in SDS as surfactant (data not shown) resulted in total algal growth inhibition.

Discussion

Potential mechanisms of observed biological responses of P. subcapitata exposed to SWNTs

Several physicochemical characteristics of SWNTs can be linked to the observed biological responses by *P. subcapitata*. Such parameters would include: (i) The generation of reactive oxygen species (ROS) and associated oxidative stress (Shvedova et al. 2003; Kagan et al. 2006), (ii) direct contact between cell and SWNTs resulting in cell membrane deformation, piercing, or uptake through passive or assisted transport (Kang et al. 2007, 2008), (iii) the levels and types of impurities associated with SWNT matrices (Shvedova et al. 2003; Kagan et al. 2006; Koyama et al. 2009), and (iv) the degree of SWNT aggregation (Wick et al. 2007). The roles of some of these parameters in the biological responses observed in this study are discussed below.

Potential ROS activity and impact on P. subcapitata growth

In this study, GSH, which is an important biomolecule for defense against heavy metals and oxidative stress in plants, was used as a specific toxicity indicator (Noctor and Foyer 1998). GSH can be considered as a representative of nucleophilic target biomolecules in algal cells. Since the enzymes glutathione-S-transferase and glutathione-synthetase are inducible by electrophilic compounds, such as SWNTs, the cellular GSH concentration would then be modulated by GSH-induction as well as by GSH-depletion caused by the presence of reactive toxicants. Therefore, disturbance of GSH metabolism is considered a toxicity parameter indicating cell damage as well as providing information about molecular modes of toxic action. Levels of GSH were investigated in 96-h algal standard growth exposure assays (Figure 4). The final GA concentrations varied from 0.04–0.17% in two sets of algal culture media containing either 0.5 or 1 ppm of SWNTs. The results showed that the increase of GA concentrations above the initially tested toxic combination of 0.023% GA and 0.5 ppm of SWNTs resulted in biomass production similar or in excess to that measured in

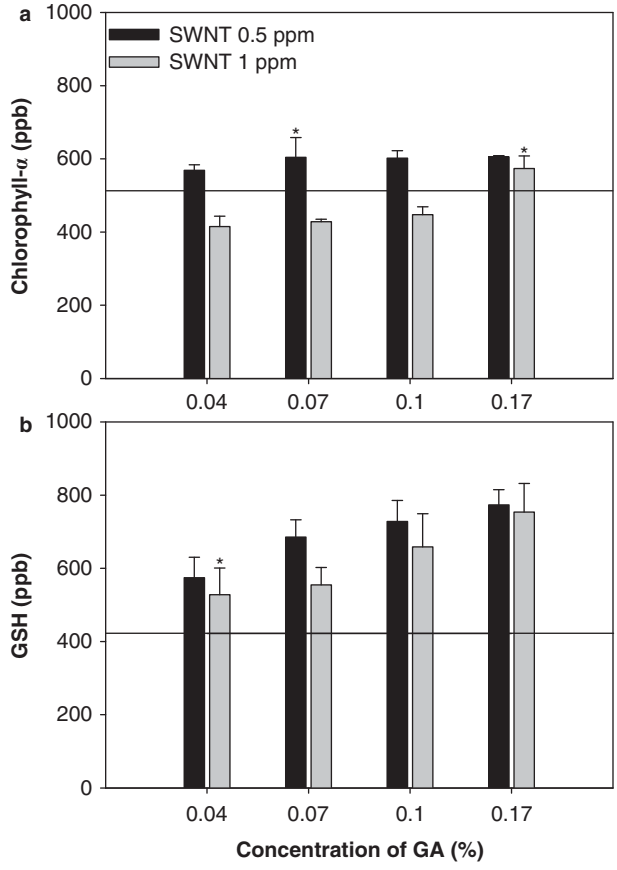

Figure 4. Effect of increasing concentrations of gum Arabic (GA) on *P. subcapitata* growth in culture media (standard 96-h chronic algal assay) containing fixed SWNT concentrations of 0.5 and 1 ppm. (a) shows changes in biomass measured as chlorophyll-*a*, and (b) illustrates the trends of glutathione (GSH) in culture media. The horizontal line represents the growth of *P. subcapitata* in control culture media (i.e., culture media containing neither GA nor SWNTs). Growth rates obtained from controls were assigned a value of 100% and biological responses measured in treated samples normalized to the control. (*) indicates the lack of significant differences as compared to controls ($\alpha > 0.05$).

control cultures (Figure 4a). Although the algal growth in culture media containing 1 ppm of SWNTs showed a slight inhibition for GA concentrations up to 0.1%, this toxicity becomes eliminated and the algal growth rate catches up with levels in control media as GA concentration reaches 0.17%.

Measurements of GSH (Figure 4b) in these culture media showed an accumulation of GSH levels, regardless of SWNT concentrations and an overall increasing trend with increasing GA concentrations. Elevated levels of GSH in SWNT-treated algal media as compared to control samples indicate the induction of GSH stimulated by oxidative stress (Lei et al. 2006), caused by SWNTs in this case. In fact, the toxicity of SWNTs has been attributed primarily to the formation of ROS and the resulting oxidative stress to living cells (Shvedova et al. 2003;

Manna et al. 2005; Kagan et al. 2006; Yang et al. 2008). However, the observed progressive build up in GSH levels with increasing concentrations of GA, which is considered a sign of oxidative stress induction (Gupta et al. 1991; May and Leaver 1993; Madamanchi et al. 1994), is also suggestive of the fact that GSH synthesis exceeds GSH depletion as GA concentrations increase in the culture media.

These results suggest that the presence of SWNTs stimulates the algal defense mechanisms as indicated by increased GSH levels. But at the same time, the increasing GA levels enhance GSH-synthesis with production rates that are higher than rates of GSH depletion through reaction with generated ROS. The overall result is a protective effect of the algal cells, and mechanisms specific to this process would include the previously reported antioxidant potential of GA in pharmaceutical studies (Abd-Allah et al. 2002; Trommer and Neubert 2005). In this case, GA in much higher concentration than GSH in culture media would behave as the primary antioxidant, allowing the accumulation of GSH produced by algal cells.

TEM observations of P. subcapitata cells grown in the presence and absence (control) of SWNTs

A previous study on SWNT-organism interactions showed that cell death could be attributed to the physical piercing of cell membranes by individual SWNTs in well-dispersed suspensions containing high concentrations up to 50 ppm (Kang et al. 2007). TEM observations of cells from the different treatments used in this study (Figure 5) showed no detectable physical cell-SWNT interactions. This could be due to the lack of sufficient contrast to distinguish between carbon-based background cell components and the carbon nanotubes. However, a look at cells exposed to the previously identified toxic combination of 0.023% GA and 0.5 ppm SWNTs (Figure 5b) in comparison with cells from control samples (Figure 5a) showed significant changes on the morphology of cell membranes. In these dividing cells, SWNT-treated cells have reduced size and deformed cell membranes (Figure 5b). These impacts could be attributable to the destructive effects of ROS due to the fact that membrane integrity is a primary target (Cabiscol et al. 2000), but one may not exclude the potential physical interaction between cell and SWNT particles, even though our TEM images do not show such occurrences. Finally, the lack of physical impacts on either controls (e.g., Figure 5a) or other samples obtained from culture media treated with GA concentrations >0.023% (data not shown) supports the finding that GA does mitigate the toxicity of SWNTs to *P. subcapitata*.

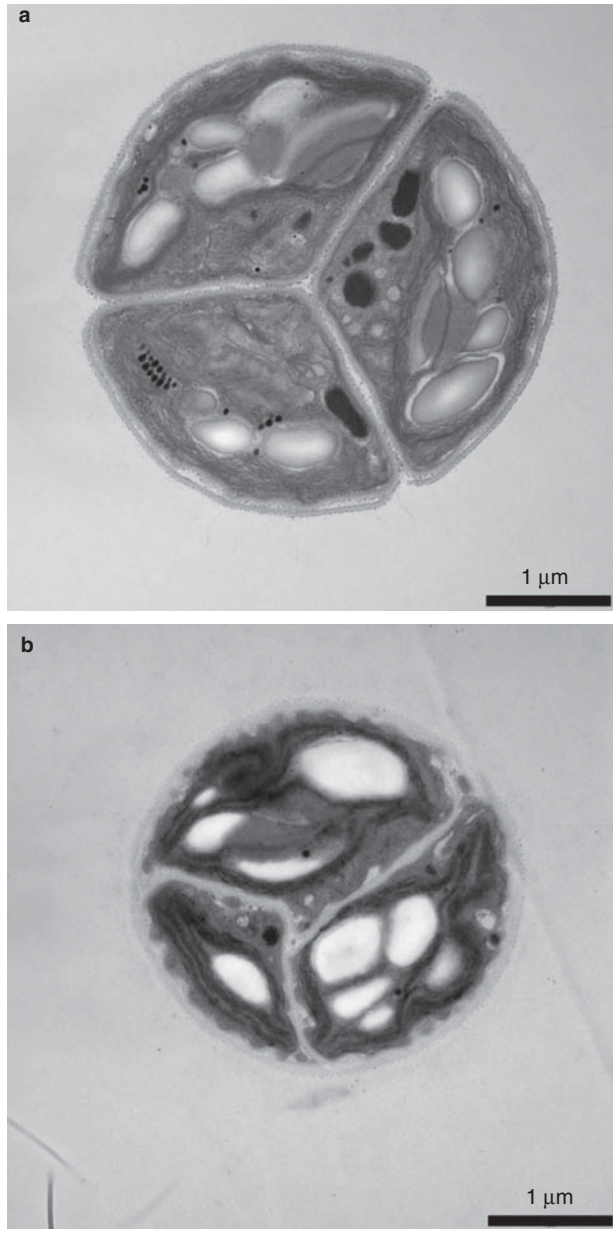

Figure 5. TEM images of *P. subcapitata* from a control sample (a) and from a culture medium containing final concentrations of 0.5 ppm and 0.023% (v/v) for SWNTs and GA (b), respectively.

State of aggregation of suspended SWNTs and impacts on algal biological responses

The analysis of the physical state of SWNTs in algal suspensions was conducted at the initial (t_0) and final (t_f) times of the algal growth experiments to gain insight into the potential mechanisms of observed cell-SWNT interactions. The sensitivity of

NIR-fluorescence spectra to environmental effects can be used to assess changes due to SWNT aggregation and quenching mechanisms during algal growth. Figure 6 shows different emission spectra obtained for sub-samples of algal suspensions containing different concentrations of GA and SWNTs collected at times t_0 and t_f. Since only individual and well-dispersed semi-conducting SWNTs emit fluorescence (O'Connell et al. 2002), these spectra are evidence that most of the SWNTs remain suspended and well-dispersed in the culture media throughout these algal exposure experiments. However, the intensity of the spectra decreased slightly for each of the tested concentrations between times t_0 and t_f.

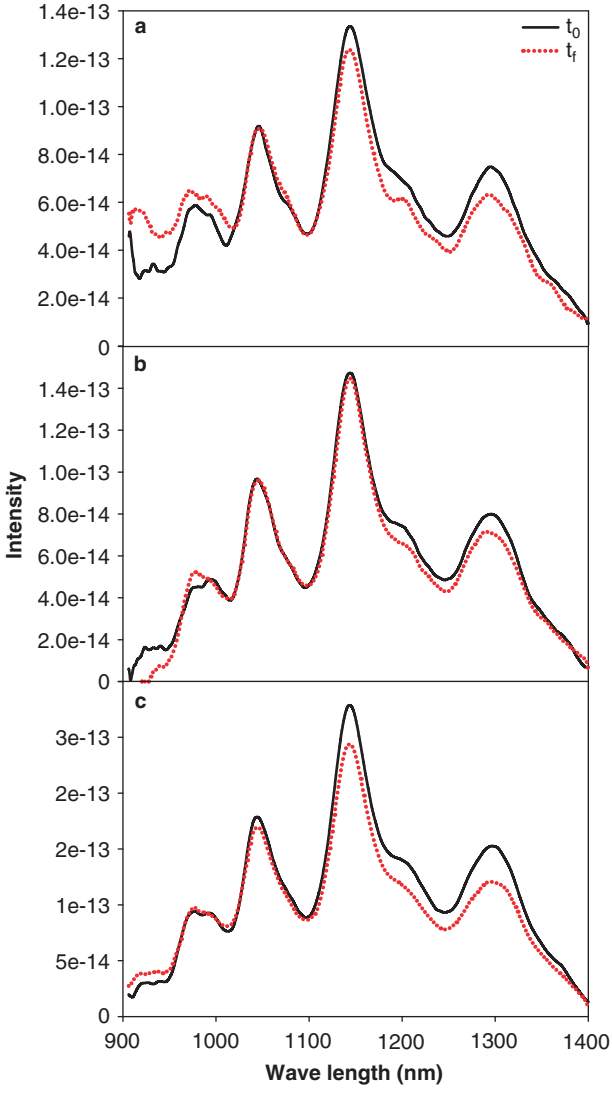

Figure 6. Near infrared fluorescence spectra of GA-SWNTs in algal suspensions with GA concentrations of (a) 0.023% with 0.5 mg SWNTs/L, (b) 0.046% with 0.5 mg SWNTs/L, and (c) 0.046% with 1mg SWNTs/L. Spectra were measured on the first (initial) and fourth (final) day of algal exposure experiments.

These changes correlate to the relative ratio of surfactant to SWNTs; photoluminescence (PL) intensity changes are more significant as this ratio decreases. The observed decrease in emission intensities suggest that one or more of the following could occur to SWNTs during algal growth: (1) Covalent or non-covalent sidewall reactions that quench SWNT-fluorescence; (2) aggregation in the suspension; or (3) removal of SWNTs by sedimentation of either SWNTs or algal cells interacting with SWNTs.

Decreases in fluorescence intensity can occur when the environment surrounding the SWNTs changes due to the replacement of surfactant molecules (GA in this case) by polymers, such as proteins (Cherukuri et al. 2004, 2006) and polysaccharides released in the culture media by growing algal cells. Therefore, it is possible that the production of dissolved organic matter (DOM) or organic exudates during algal growth could result in the formation of DOM-coated SWNTs. However, surfactant replacement is typically accompanied by characteristic solvatochromic peak shifts associated with the new environment surrounding the nanotubes (Moore et al. 2003; Cherukuri et al. 2004, 2006). In this study, only a 1–2 nm solvatochromic shift in peak position was observed. This small difference indicates that only a very small amount of algal produced DOM could possibly replace GA molecules surrounding the SWNTs. These changes could indicate some changes to the surface coverage of the surfactant, which reduces the ability of the layer to prevent fluorescence quenching mechanisms (Wang et al. 2008; Silvera-Batista et al. 2009). However, any changes to the surfactant structure must be minimal since exposure to higher dielectric environments are expected to decrease the PL intensity dramatically (Silvera-Batista et al. 2010).

It is also possible that the sidewalls of the nanotubes become functionalized during algal growth, reducing the PL intensity. However, Raman spectra would indicate structural changes to the carbon atoms on the sidewalls of SWNTs associated with covalent functionalization. The D-peak (at about 1300 cm^{-1}) and G-peak (at about 1600 cm^{-1}) on such spectra correspond to phonon modes associated with sp^3 and sp^2 carbon atoms of SWNTs, respectively. In this study, spectra obtained for different GA concentrations (0.023% and 0.046% with 0.5 ppm SWNTs) at both t_0 and t_f exhibit nearly identical D/G peak ratios (see Figure 7), suggesting that nanotubes do not undergo significant chemical changes, such as oxidation or sidewall functionalization. This is because high D/G ratios are obtained only when covalent bonds are formed on SWNT surfaces causing the phonon modes of sp^2 carbon atoms to shift to sp^3 phonon modes (Jorio et al. 2003).

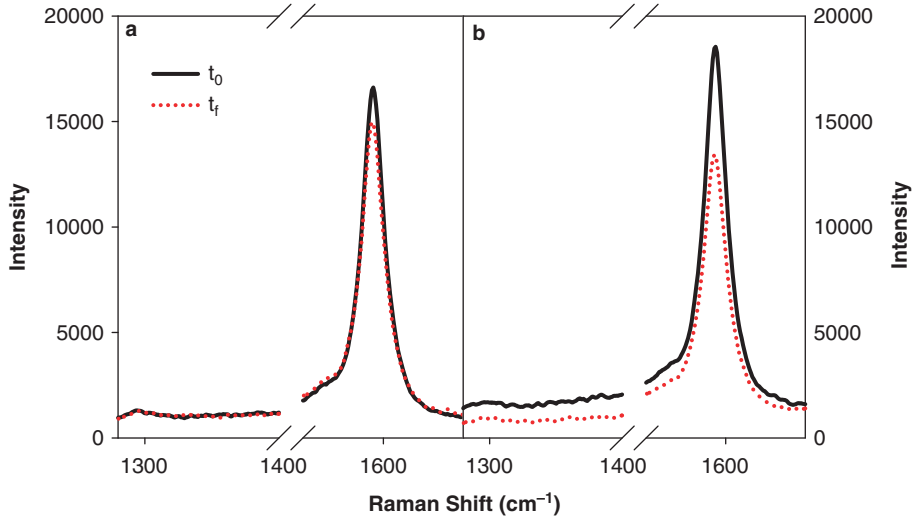

Figure 7. Raman spectra of GA-coated SWNTs (0.5 ppm) in algal suspensions measured on the first (t_0) and fourth (t_f) day of algal exposure experiments. (a) 0.023% GA; (b) 0.046% GA.

The decrease in the intensity of the PL (see Figure 6) and G-peak between t_0 and t_f (Figure 7) indicate that some SWNTs could be removed from the algal suspensions. Such sedimentation or removal of SWNTs could occur following the aggregation of a small fraction of SWNTs present in the suspension. This is important as previous exposure studies investigating the effects of the degree and kind of aggregation of SWNTs on cells have linked these parameters to biological responses in human MSTO-21 cells (Wick et al. 2007). Finally, although the above discussion suggested that complete exchange of the surfactant with DOM on SWNT surfaces is unlikely, the adsorption of even a small amount of DOM could alter the surfactant structure around the nanotubes (Wang et al. 2008) or change the surface charges (Summers and Roberts 1988), disrupting the mechanism that disperses SWNTs. The induced progressive aggregation of SWNTs in such cases would promote removal from solution by sedimentation and possibly alter the biological response of the tested model organisms as well. However, considerable aggregation of SWNTs would be expected to have significant decreases in PL intensity, which is not observed.

Therefore, the spectroscopy data suggest that the SWNTs in the suspension are similar in surfactant coverage and aggregation state throughout algal growth. However, the fact that the PL intensity changes are correlated to the relative ratio of surfactant to SWNTs indicates that both of these factors could occur to a limited extent during algal growth. Although aggregation and surfactant changes cannot be ruled out, it is clear that these effects have minimal influence on the SWNTs during algal growth at all SWNT concentrations. Therefore, our study suggests that well-dispersed SWNTs in aqueous suspensions can result in negative biological effects at concentrations >1 ppm, which is in contrast to previous studies that found that aggregation was responsible for toxicity (Wick et al. 2007).

Impacts of impurities found on SWNTs on algal growth

The biological response of organisms exposed to SWNTs can also be affected by the load of impurities common to carbon nanotubes (Shvedova et al. 2003; Kagan et al. 2006; Smart et al. 2006; Koyama et al. 2009). For SWNTs used in this study, the load of impurities amounted to 13.4 weight % as measured by TGA, and ICP-AES analyses detected only iron in levels above the instrument's detection limits (*ca.* 17 ppm). Previous research has linked Fe-impurities to toxicity due to its role in the formation of free radicals and ROS (Shvedova et al. 2003; Nel et al. 2006), and the activity of ROS tends to be proportional to the concentration of Fe impurities in SWNT suspension (Kagan et al. 2006). Moreover, the continuous exposure of algal cultures to light certainly favors different photochemical reactions, such as the Fenton reaction. With regard to the experimental results obtained in this study, ROS production induced by Fe-impurities is possible, and if so, the previously reported antioxidant potential of GA (Abd-Allah et al. 2002; Trommer and Neubert 2005) could play a role in the observed decreasing toxicity trend with increasing GA concentrations in culture media.

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