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RESEARCH PAPER

Activity inhibition on municipal activated sludge by single-walled carbon nanotubes

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Abstract The objective of this study was to evaluate the respiratory activity inhibition of activated sludge used in a typical wastewater treatment plant by single-walled carbon nanotubes (SWCNTs) with different length and functionality. Four types of SWCNTs were evaluated: short, functionalized short, long, and functionalized long. Based on the effective concentration (EC50) values obtained, we determined that functionalized SWCNTs resulted in a higher microbial respiratory inhibition than non-functionalized nanotubes, and long SWCNTs gave a higher microbial respiratory inhibition than their short counterparts. Among the four types of SWCNTs studied, functionalized long exhibited the highest respiration inhibition. Scanning electron microscopy imaging indicates that the long SWCNTs dispersed more favorably after sonication than the short variety. The findings demonstrated that the toxicity of CNTs (exhibited by respiratory inhibition) is related to their physical properties; the length and functionality of SWCNTs affected the toxicity of SWCNTs in a mixed-cultured biologic system.

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Introduction

Discovered in the early 1990s, carbon nanotubes (CNTs) are single sheets of graphite rolled into tubes with diameters no larger than low nanometers (Bastús et al. 2008). The physical and chemical properties of carbon nanotubes have led to a wide variety of new research ventures and possible applications. As manufacturing and research endeavors continue to expand (Baughman et al. 2002), environmental impact analysis needs to stay alongside or ahead to limit any chances of adverse impacts on humans and the environment. Carbon nanotubes are known to have a negative impact on cell viability (Pogodin and Baulin 2010). An in vitro assay showed that *Staphylococcus aureus* and *Staphylococcus warneri* bacteria could not grow over the CNTs films (Narayan et al. 2005). Antimicrobial activity of single-walled carbon nanotubes (SWCNTs) was observed after *E. coli* were exposed to SWCNTs (Kang et al. 2007, 2008). Bottini et al. (2006) reported that 80 % of the cells exposed to oxidized CNTs at the concentration of 400 μg/L were killed.

The exact causes of microbial toxicity due to interactions with CNTs are still in debate. It could be a single factor, such as cell penetration or oxidative stress via contact, or a combination of many effects. Several studies have modeled and examined cell penetration and destruction by nanotubes. Liu et al. (2009) described them as "nano darts", using their small size to puncture and destabilize the outer membrane wall of cells, leading them to lysis and death. Oxidative stress has also been reported to occur, with a possible result of cell death. Interactions between the cells and the surface of the nanotubes can oxidize molecules vital to the cells (Bello et al. 2009). Nanotubes have also been shown to have the capacity to enter and oxidize DNA, disrupting the protein chains and resulting in cell failure during mitosis (Firme and Bandaru 2010). Any of these processes are enough to warrant cell toxicity, but any chemicals attached to the nanotubes (e.g., functionalization) can cause a similar effect as well (Zhao and Liu 2012).

The physical properties of carbon nanotubes are known to hinder efforts to disperse them in fluids. When placed in a polar solution such as water, the individual pristine nanotubes will aggregate together to form larger clumps, many of which will settle out. This aggregation lowers overall contact with cells and reduces any impact of cell penetration (Liu et al. 2009). Dispersing these raw carbon nanotubes is often assisted with the addition of chemicals and surfactants (Hyung et al. 2007; Manivannan et al. 2007; Saleh et al. 2008; Wang et al. 2008) or natural organic matter (Hyung et al. 2007). Hyung et al. (2007) commented that the addition of natural organic matter to solutions of carbon nanotubes allowed them to remain in suspension for over a month. Recently, physicochemical properties have been recognized as important factors that can greatly affect nanoparticles toxicity. Comparative studies have shown that SWCNTs exhibited higher toxicity as compared to multi-walled carbon nanotubes (MWCNTs). Jia et al. (2005) reported the following order of toxicity on a per-mass basis in alveolar macrophages: SWCNTs > MWCNTs > quartz > fullerenes. Kang et al. (2009) also reported that the degree of toxicity was dependent on the type of nanomaterial (SWCNTs were more toxic than MWCNTs) and bacterial species (*Bacillus subtilis* was less sensitive than Gram-negative species). Oxidized CNTs were found to be more toxic than unoxidized, pristine CNTs (Bottini et al. 2006). Although SWCNTs are generally concluded as being more toxic, there is a lack of study evaluating how the length and functionality of SWCNTs will impact their toxicity.

Nanomaterials are still a relatively new class of substances, and as such should be treated with caution. A complex environmental system such as the activated sludge wastewater treatment process has diverse microbial communities with cells free swimming or embedded in flocs (Luongo and Zhang 2010); it could complicate microbial response to toxicity posed by nanoparticles (Kang et al. 2009). This study aimed to examine the impact of the length and functionality of SWCNTs on the respiratory activity of the activated sludge used in wastewater treatment facilities and quantify such effect through respiratory inhibition. Activated sludge respiratory activity inhibition test had been used by several researchers (Henriques and Love 2007; Luongo and Zhang 2010) to examine the toxicity response of activated sludge to chemical toxins. This test measures the overall metabolic respiratory activity. Thus, the negative effects on the biologic activity can be taken as the result of metabolic inhibition due to the presence of toxins (Gendig et al. 2003).

Materials and methods

Nanotube preparation

Four types of SWCNTs were purchased from Cheaptubes, located in Brattleboro, VT. They were short SWCNTs, carboxylic functionalized short SWCNTs, long SWCNTs, and carboxylic functionalized long SWCNTs (their characteristics are shown in Table 1). SWCNTs were chosen in this study because of their demonstrated toxicity through pure culture studies (Kang et al. 2007); different types of SWCNTs were chosen in order to evaluate how the length and functionality of CNTs may affect their potential toxicity in a mixed-cultured environmental system.

All SWCNTs were sonicated to help them disperse prior to studying their effect on the respiratory activity of the activated sludge used in wastewater treatment. Each type of SWCNTs was prepared by mixing a measured amount of SWCNTs, 5 mL of concentrated synthetic sewage feed (see "Respiration inhibition

Table 1 Characteristics of single-walled carbon nanotubes used (all with purity >90 % and ash content <1.5 %)

SWCNTs	Type	Outer diameter (nm)	Length (μm)	Specific surface area (m^2/g)	Functional content (–COOH, %)	Additional MWCNT content (%)
Short	Non-functionalized	1–2	0.5–2	>407	0	5
	Functionalized	1–2	0.5–2	>407	2.73	0
Long	Non-functionalized	1–2	5–30	>407	0	0
	Functionalized	1–2	5–30	>407	2.73	0

MWCNTs Multi-walled carbon nanotubes

Table 2 Preparation of SWCNT samples prior to sonication

Sample number	SWCNTs (g)	Concentrated synthetic feed (mL)	DI water (mL)	SWCNT concentration during sonication (mg/L)	Resulting nanotube concentration in the activated sludge jar tester (mg/L)[a]
1	0.05	5	75	625	100
2	0.15	5	75	1,875	300
3	0.25	5	75	3,125	500

[a] See Table 3

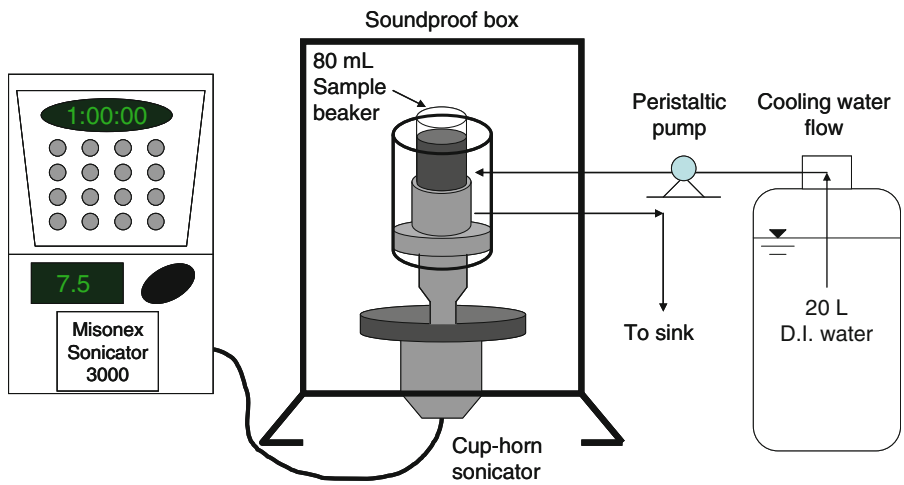

Fig. 1 Sonication system setup

test" section) and 75 mL of DI water (see Table 2). Each mixture was briefly stirred by hand using a glass stir rod and then sonicated at a dial setting of 7.5 for 2 h, pulsing ON and OFF for 30 s intervals using a Misonex Sonicator 3000 with cup horn. Iced water was pumped through the system to insure steady temperatures. The setup of the sonication system is shown in Fig. 1. Following sonication, the mixtures were placed into a refrigerator for storage. Prior to use in the experiment, each mixture was stirred by hand to eliminate any nanotubes that had caked onto the bottom of the beaker and sonicated again for 10 min at a dial setting of 7.5.

Respiration inhibition test

Respiratory inhibition of the bacteria by SWCNTs was examined using a slightly modified protocol by EPA

Table 3 Experimental design of six-beaker jar testers

	Sonicated SWCNTs (mL)	Concentrated synthetic feed (mL)	Concentrated mixed liquor (mL)	DI water (mL)	3,5-Dichlorophenol (mL)[a]	Final concentration (mg/L)
SWCNTs[b]	75	5	200	220	0	100
	75	5	200	220	0	300
	75	5	200	220	0	500
Reference #1	0	5	200	290	5	5
Reference #2	0	5	200	285	10	10
Reference #3	0	5	200	270	25	25
Control #1	0	5	200	295	0	0
Control #2	0	5	200	295	0	0

[a] From 3,5-dichlorophenol stock solution which is 500 mg/L

[b] See Table 2 for the preparation of each SWCNTs mixture

(EPA 1996), and the detailed procedure was presented previously (Luongo and Zhang 2010).

Synthetic feed was used as a food source for the bacteria to insure the respiration results were not affected by starvation. The composition of the synthetic feed is as follows: 16 g peptone, 11 g meat extract, 3 g urea, 0.7 g NaCl, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 2.8 g K_2HPO_4 mixed into 1 L of DI water. This concentrated feed was added to the sludge for overnight storage (the amount of which was determined to keep the food-to-microorganism ratio consistent), as well as in each jar tester during experimentation.

One day prior to experimentation, 10–12 L of activated sludge was collected from the Lowell Regional Wastewater Utility in Lowell, Massachusetts. The sludge was immediately returned to the UMass Lowell laboratory and aerated. An initial chemical oxygen demand (COD) and mixed liquor suspended solids (MLSS) reading were taken following the standard methods (APHA et al. 1998). The sludge was then decanted and washed three times with tap water to remove dissolved organic matter and allowed to settle. A second MLSS measurement was taken, and the sludge was concentrated to 4,000 mg MLSS/L (i.e., concentrated mixed liquor). After concentration, a second COD measurement of the sludge was taken. Using the obtained data, synthetic feed was added to the sludge as food, with the volume varying to insure the food/microorganism ratio was similar to the initial sample during storage.

On the day of the experiment, two six-beaker jar testers were run simultaneously. Each beaker was prepared according to Table 3 with a final volume of 500 mL and allowed to run for 3 h each. For each type of SWCNT studied, three different SWCNT concentrations were prepared (100, 300, and 500 mg/L), and each concentration was run in duplicate. Conditions for each beaker were kept identical using air flow meters to hold aeration at 1 L/min and mixed at 90 rpm. The beakers were staggered 15 min apart to allow for preparation and dissolved oxygen (DO) readings. At the end of each beaker's 3-h run, the DO levels were recorded over a 10 min span at 30 s intervals using a YSI Model 52CE meter and probe, and the results were reported in units of mg O_2/L-h.

A reference chemical, 3,5-dichlorophenol, was used to insure the bacteria in the sludge were not already stressed or damaged. Three references with 5, 10, and 25 mg/L of concentrations were run along with the samples. The EC50 of the reference must fall within 5–30 mg/L, EC50 being the effective concentration of toxins that produce a 50 % inhibition rate. Two controls without CNTs and references were run as well, one at the beginning of the experiment and the other at the end of the 3 h contact time. The respiration rates from the two controls must fall within 15 % of each other (Eq. 1). If either the reference or the controls were out of range, the experimental results were considered invalid.

$$\%\ \text{Difference of controls} = \left|\frac{R_{C1} - R_{C2}}{(R_{C1} + R_{C2})/2}\right| \times 100\,\% \quad (1)$$

where R_{C1} is the respiration rate of control 1 (mg/L h). R_{C2} is he respiration rate of control 2 (mg/L h).

Respiration rates of the activated sludge mixed with SWCNTs were determined by comparing their DO

usage rates to the two controls and were calculated by the use of Eq. 2.

$$\% \text{ Inhibition of samples } = \left[1 - \frac{2R_S}{R_{C1} + R_{C2}}\right] \times 100\,\% \quad (2)$$

where R_S respiration rate of the activated sludge mixed with SWCNTs (mg/L h).

From the % inhibition calculated, a linear relationship between % inhibition and SWCNTs concentrations can be established. Then, the respective EC50 (50 % inhibition) of each SWCNTs can be calculated and compared to demonstrate their potential toxicity.

SEM imaging

A scanning electron microscopy (SEM) was used to take images after SWCNTs were sonicated to demonstrate the level of dispersion. A JEOL JSM-6390 using a tungsten electrode was used. Samples were prepared a day in advance for imaging to insure proper dehydration. One mL of the sample was taken from the CNT mixture (see Table 2 for details) after sonication and diluted to 300 mg/L. The prepared SEM sample was then placed on an OSMONICS AcetatePlus filter (or polycarbonate membrane filter with 0.4 μm pore size in Figs. 6, 7), and washed with 1 mL 2.5 % glutaraldehyde and allowed to set in a refrigerator for two hours. Following that, the sample was washed with a 0.1 M phosphate buffer and immersed in increasing concentrations of ethanol to remove any moisture. The dehydrated sample was then stored in a desiccator until use. Prior to imaging, the samples were gold-coated using a Denton Vacuum Desk IV Sputter Coater at 25 % power for 2 min to provide a better imaging surface.

Results and discussion

A SEM image was taken for each 300 mg/L nanotube sample after sonication to demonstrate the dispersion of CNTs (Figs. 2, 3, 4, 5). By examining the SEM images, it can be seen that long SWCNTs were more readily dispersed than short SWCNTs, and they appeared as loose aggregates with an average diameter of approximately 20 nm after sonication. This number is higher than the manufacturer-reported outer diameter (1–2 nm) due to the nature of CNTs' aggregation tendency in solution.

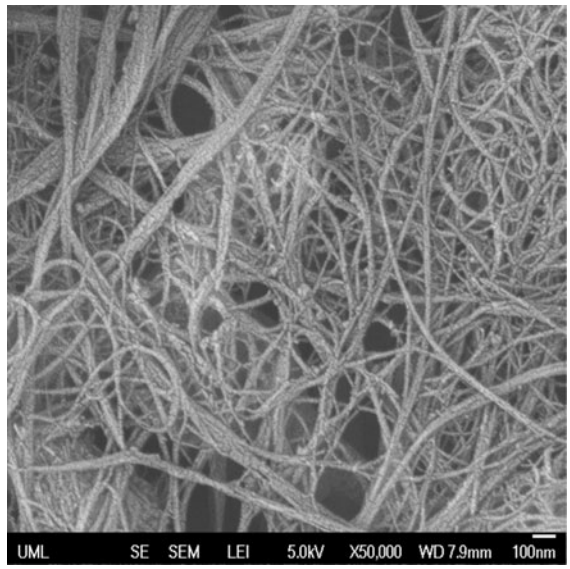

Fig. 2 SEM of functionalized long SWCNTs (300 mg/L) after sonication

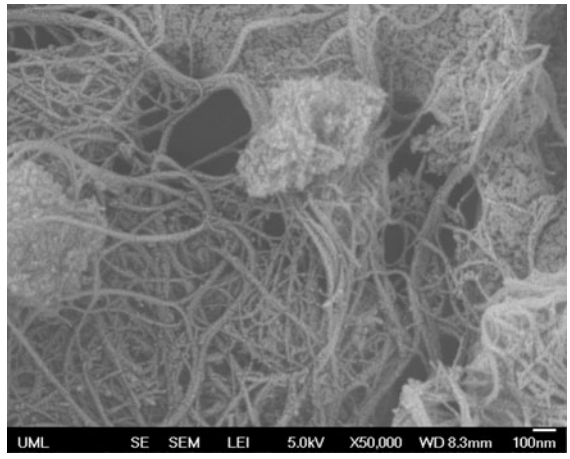

Fig. 3 SEM of long SWCNTs (300 mg/L) after sonication

Respiratory inhibition results for all four CNTs are listed in Table 4. These EC50 values indicate the concentrations that would produce a 50 % reduction in oxygen use by the microorganisms in the activated sludge. Lower EC50 values show that less of the nanotubes are required to produce the 50 % inhibitory effect, meaning that smaller EC50 values indicate a higher inhibition (more toxic effect) from the chemical added.

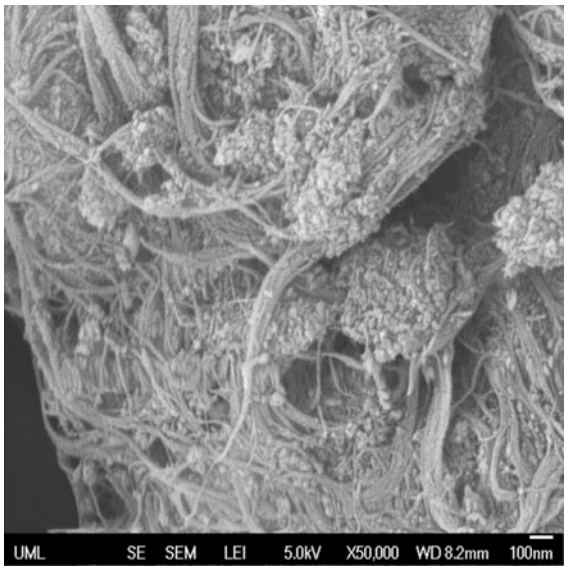

Fig. 4 SEM of functionalized short SWCNTs (300 mg/L) after sonication

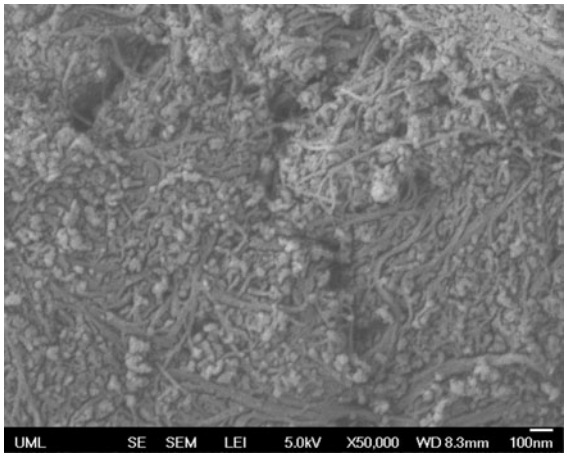

Fig. 5 SEM of short SWCNTs (300 mg/L) after sonication

Analysis of this data leads to two conclusions. First, long SWCNTs (5–30 μm) are more inhibitory than the short SWCNTs (0.5–2 μm). Second, functionalized SWCNTs are more inhibitory than the non-functionalized SWCNTs. The non-ideal R^2 values reflect the complex nature of a mix-cultured microbial system.

The role of nanotube length in governing toxic responses is of particular interest because of their length similarity to asbestos. Long asbestos fibers are known to generate chronic inflammatory responses. In a recent study, Poland et al. (2008) injected long (>10–20 μm), short (<5 μm) MWCNT, and asbestos fibers into the abdominal lining of mice. Significant asbestos-like scarring and inflammation of the abdominal lining, which are considered to be a precursor to mesothelioma, were found only from the exposure to the long nanotubes. A separate research group studying the genotoxicity of SWCNTs using human cells came to a similar conclusion (Manshian et al. 2012). They found that long SWCNTs (5–30 μm) caused more chromosomal damage than the short (1–3 μm). These findings all point out that physiology of SWCNTs such as length can play a significant role in inducing microbial toxicity.

The results illustrated that functionalization also played a significant role in the respiratory inhibition induced by SWCNTs. Carboxylic functionalized SWCNTs exhibited higher respiration inhibition (EC50 = 1,656 mg/L for the long) toward the activated sludge microbial system comparing to the non-functionalized SWCNTs. Bottini et al. (2006) reported that oxidized CNTs were more toxic than unoxidized, pristine CNTs. The attached carboxylic functional groups can help SWCNTs become more dissolved or dispersed in water (Shieh et al. 2007) which will increase their hydrophilicity (or decrease their hydrophobicity). Henriques and Love (2007) reported that the penetration of the toxin into the floc matrix is reduced if the toxin is hydrophobic, suggesting that more hydrophilic toxins (e.g., functionalized SWCNTs) perhaps can penetrate deeper into the floc matrix and cause more toxicity toward the microorganisms present in the flocs.

Two SEM images were obtained for the mixture of carboxylic functionalized long SWCNTs and activated sludge flocs. Figure 6 was obtained shortly after the CNTs was added into the activated sludge reactor. It shows cohesive and compact aggregates between CNTs and activated sludge flocs were formed as soon as the CNTs were added, illustrating the interaction between the flocs and CNTs was immediate. At the end of the 3-h contact time, Fig. 7 was obtained, and the CNTs appeared to be even better dispersed; in addition, it again demonstrates that some rod-shaped bacteria and small flocs are in direct and close contact with CNTs. These images suggest mechanical mixing and longer contact time could help disperse CNTs and as a result, bring CNTs closer to the microorganisms within the flocs and create more physical contact with the microorganisms in the activated sludge and induce

Table 4 EC50 values for different types of SWCNTs studied

	CNTs type	Reference 3,5-dichlorophenol EC50 values (mg/L)	% Inhibition vs. CNT concentration	CNTs EC50 values (mg/L)
Long SWCNTs	Functionalized	9.06	$y = 0.0302x$ $R^2 = 0.66$	1,656
	Non-functionalized	11.8	$y = 0.0172x$ $R^2 = 0.64$	2,907
Short SWCNTs	Functionalized	15.13	$y = 0.0102x$ $R^2 = 0.71$	4,902
	Non-functionalized	11.52	$y = 0.0063x$ $R^2 = 0.80$	7,937

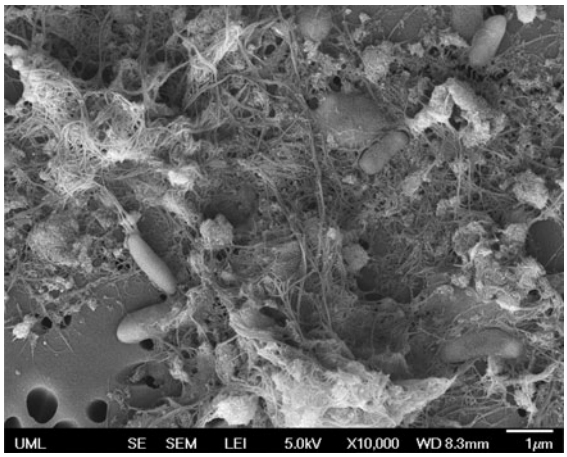

Fig. 6 Long functionalized SWCNTs after brief contact with activated sludge

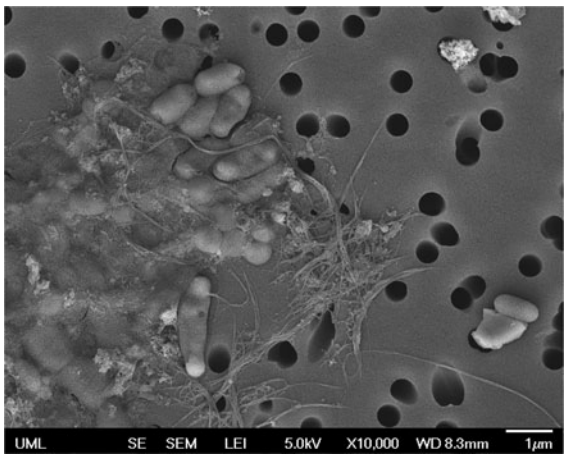

Fig. 7 Long functionalized SWCNTs after 3 h of mixing with activated sludge

more toxicity. Luongo and Zhang (2010) reported similar findings with the same experimental setup.

In addition to length and functionalization of CNTs, the dispersion of CNTs could also affect their microbial toxicity. The obtained higher respiration inhibition from long SWCNTs seems to suggest that more dispersed CNTs could induce more microbial toxicity. The level of dispersion impacts the degree of contact between the microorganism and nanotubes, and direct contact between the microorganism and CNTs has been found to be critical to induce cell toxicity (Kang et al. 2008). More contact would allow individual nanotubes to penetrate the cells and flocs more easily (acting as ''nano darts'') (Liu et al. 2009). Other mechanisms such as "piercing", cell membrane damage, DNA damage, oxidative stress, or a combination of the above have also been suggested and could contribute to the results obtained. However, more research is needed to provide further evidence for the mechanisms involved in the observed respiratory activity inhibition.

The EC50 values for the reference chemical used (3,5-dichlorophenol) were found to be between 9 and 15 mg/L (Table 4), suggesting that the potential for SWCNTs to induce microbial toxicity to an activated sludge system is very low (much more SWCNTs would be required in order to induce 50 % respiration inhibition). However, care should be taken into account that each reported toxicity value is related to the respective experimental conditions used. The toxicity of CNTs will largely depend on the manufacturers, their concentrations, their physical form, length, functionality, attached molecules (Bottini et al. 2006), and the microbial system studied. Depending on the microbial systems studied,

consideration should be given to the amount of extracellular polymeric substances (EPS) present in bacterial flocs. Luongo and Zhang (2010) showed that EPS can shield the microorganisms from the toxicity present in the surrounding solution. In a system that EPS is lacking, the potential for SWCNTs to impose toxicity can be much higher. Slight differences in the physio-chemical characteristics of nanomaterials are sufficient to induce variations in the CNTs toxicity (Manshian et al. 2012). The duration of the study and preparation of the nanomaterial would also affect the results, e.g., the sonication technique used in this study greatly affected the dispersion of CNTs. As shown in Figs. 2, 3, 4, 5, CNTs were not totally dispersed, and consequently, it affected the EC50 values obtained. Therefore, comprehensive and through toxicity study is still needed to fully understand the long-term toxicity effect of CNTs in all microbial systems.

Conclusions

As the prevalence of carbon nanotubes increases, environmental examination and oversight must increase as well.

A respiration inhibition test was used to analyze the toxicity effects of SWCNTs on the microorganisms used in municipal activated sludge wastewater treatment process. The following conclusions have been obtained.

- SWCNTs induced a reduction in bacterial respiratory activity when carbon nanotubes and bacteria are allowed to contact each other.
- Carboxylic functionalization of the nanotubes resulted in a higher degree of bacterial activity reduction, or an increase in the possible toxicity.
- The length of the nanotubes affected the ability to disperse discreet tubes in solution, with longer tubes more readily dispersing and resulting in a higher degree of bacterial activity reduction.

This finding demonstrates that the toxicity of CNTs (exhibited by respiration inhibition) is related to their physical properties; the length and functionalization affected the toxicity of SWCNTs in a mixed-cultured biologic system.

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