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Toxicity of carbon nanotubes to the activated sludge process

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ABSTRACT

The discharge of carbon nanotubes (CNTs) from industrial waste or disposal of such materials from commercial and/or domestic use will inevitably occur with increasing production and enter into wastewater treatment facilities with unknown consequences. Therefore, a better knowledge of the toxicity of CNTs to biological processes in wastewater treatment will be critical. This study examined the toxicity of multi-walled carbon nanotubes (MWCNTs) on the microbial communities in activated sludge. A comparative study using the activated sludge respiration inhibition test was performed on both unsheared mixed liquor and sheared mixed liquor to demonstrate the potential toxicity posed by MWCNTs and to illustrate the extent of extracellular polymeric substances (EPS) in protecting the microorganisms from the toxicity of CNTs. Respiration inhibition was observed for both unsheared and sheared mixed liquor when MWCNTs were present, however, greater respiration inhibition was observed for the sheared mixed liquor. The toxicity observed by the respiration inhibition test was determined to be dose-dependent; the highest concentration of MWCNTs exhibited the highest respiration inhibition. Scanning Electron Microscopy (SEM) images demonstrated direct physical contact between MWCNTs and activated sludge flocs.

1. Introduction

Carbon nanotubes (CNTs) are considered a novel material with growing commercial application due to their unique properties. CNTs are tubular, graphite sheets consisting of sp^2 carbon bonds typically with diameters of 1.4 nm and lengths in the microns [1]. The large surface area to volume ratio, tensile strength, and electrical properties make CNTs ideal components of composites, sensors and probes, and energy storage devices, such as fuel cells. By 2010, the value of the carbon nanotube market is estimated to be over $1.9 billion [2]. In the United States alone, $1.5 billion has been invested into nanotechnology in 2008 [3].

As a result of the increasing development of CNT application and production, there arises an increasing concern regarding the risks to biological processes and systems. Numerous studies have investigated the human health implications of nanomaterials [4,5]. Only recently have researchers begun to study the potential ecological risks (e.g., wildlife such as largemouth bass [6]) and impacts of nanoparticle release to the environment. Microbial toxicity of CNTs has been demonstrated under pure culture conditions. An *in vitro* assay showed that *Staphylococcus aureus* and *Staphylococcus warneri* bacteria could not grow over the CNTs films [7]. Antimicrobial activity of SWCNTs was observed after *Escherichia coli* were exposed to SWCNTs [8,9]. In another study, Bottini et al. [10] reported that 80% of the cells exposed to oxidized CNTs at the concentration of 400 μg/L were killed.

Several potential mechanisms of the toxicity imposed by CNTs have been reported. Carbon nanotubes may act as "nanosyringes" and cause highly localized disruption of bacterial cell walls and membranes [8]. Direct contact of CNTs with bacteria was necessary and caused damage in cell membrane [9]. In addition, cellular exposure to CNTs may result in the generation of reactive oxygen species that can damage DNA, proteins, and membranes [11,12]. Recently, physicochemical properties have been recognized as important factors that can greatly affect nanoparticles toxicity. Higher toxicity was observed when MWCNTs were short and dispersed in solution when tested on bacterial cultures [13] and blood serum [14]. Oxidized CNTs were more toxic than un-oxidized, pristine CNTs [10]. The results on the toxicity effects of catalytic metal residues in unpurified CNTs are limited and inconclusive. When Fe was used as a catalyst to synthesize SWCNTs, greater oxidative stress and permeation to the human keratinocyte cells was observed [15]. In contrast, Bello et al. [14] found no significant associations between soluble metal content and biological oxidative damage using human blood serum. Kang et al. [13] found that Fe content in MWCNTs did not correlate to bacterial cell membrane damage.

Recent studies have highlighted the need to move beyond pure culture systems in order to assess the true risk posed by CNTs to

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environmental systems (e.g., [16]). However, so far, only a handful of studies have investigated the impact of nanomaterials on complex microbial systems. Choi et al. [17] investigated the impact of nanosilver on nitrifying bacteria and observed inhibited respiration of 86% at 1 mg/L, much greater than for silver ions and silver chloride colloids (42% and 46%). Limback et al. [18] reported the removal efficiency of cerium oxide nanoparticles in a pilot wastewater treatment plant; they found that agglomeration and absorption to the activated sludge was significant and concluded that the surface charge of the activated sludge flocs and dispersion methods used play a key role in the removal efficiency of the nanoparticles from wastewater treatment plants. Kang et al. [16], Yin et al. [19], and Yin and Zhang [20] are probably the only papers that studied the interaction of CNTs with a wastewater sample, noting that Yin's team simulated the activated sludge process itself and Kang's team used wastewater effluent as a test medium. Kang et al. [16] showed that CNTs were toxic to bacteria in wastewater effluent. Yin et al. [19] and Yin and Zhang [20] showed that SWCNTs improved sludge settleability. But SWCNTs did not affect the performance of a continuous reactor (measured by chemical oxygen demand (COD) removal and effluent total suspended solids) [20]. Greater COD was removed in a batch-reactor study due largely to adsorption by SWCNTs [19].

Extracellular polymeric substances (EPS) in the activated sludge come from the natural secretions of bacteria, cell surface material shedding, cell lysis, and hydrolysis products from wastewater [21]. Studies have revealed that microorganisms in the activated sludge process may be protected from various toxins by EPS [22,23]. The toxins that have been studied include octanol, cadmium, lead, nickel, N-ethylmaleimide and cyanide [22,23]. The degree of the protective ability of EPS depends on the nature of the toxins [23]. The penetration of the toxin into the floc matrix is believed to be reduced if the toxin is hydrophobic, leading to its greater interaction and sorption to the hydrophobic floc structure. The interaction could prevent the toxic compounds from readily being exposed to the microbial communities present in the flocs [23]. However, the role of EPS in protecting the microorganisms in the activated sludge when exposed to nanoparticles has not been studied.

A complex environmental system such as the activated sludge wastewater treatment process has diverse microbial communities with cells free swimming or embedded in flocs. Furthermore, its characteristics such as elevated concentrations of suspended and dissolved organic matter could complicate microbial response to toxicity posed by nanoparticles [16]. Therefore, more research is urgently needed to understand the impact of CNTs on a complex environmental system. The objective of this study was to evaluate the potential toxicity posed by MWCNTs on the microbial communities in the activated sludge by using a respiration inhibition test and the role of EPS in protecting the microorganisms when the activated sludge is exposed to MWCNTs.

The activated sludge respiration inhibition test measures the overall metabolic respiratory activity. Thus, the negative affects on the biological activity due to the presence of toxins are the result of metabolic inhibition [24]. MWCNTs were chosen for this study because of their wide industrial applications [1] and reasonable pricing.

2. Materials and methods

2.1. Preparation of MWCNTs

We used commercially available MWCNTs (Sigma–Aldrich). The manufacturer reports that the sample is over 90 mass% MWCNTs, with a powder density of 2.1 g/cm^3 at 25 °C. It is also reported that the MWCNTs have on average 10–15 nm outer diameter, 2–6 nm inner diameter, and 0.1–10 μm in length. The reported impurities are 2.3% Al and 1.9% Fe trace metal with no amorphous carbon present.

Four concentrations of MWCNTs in distilled water were prepared. The final concentrations for the 500 mL volume of each reactor were: 0.64, 1.44, 2.16, and 3.24 g/L; selection of these concentrations was based on preliminary tests performed in the laboratory in order to achieve a dose-response of the impact of MWCNTs on the respiratory inhibition to the microbial communities in the activated sludge. The MWCNTs were sonicated to achieve better dispersion by using an ultrasonic cup horn (Sonicator 3000 Ultrasonic Liquid Processor, Misonix Incorporated). The sonication process began by mixing each concentration of MWCNTs for 30 min in distilled water with stirbars, sonicating the samples for 1 h, followed by mixing with stirbars for approximately 48 h, and then sonicating for 10 min before input into the mixed liquor. Sonication power outage was maintained at 75 W.

2.2. Field sampling and sample preparation

Fresh activated sludge was obtained from the aeration tank of the Lowell Wastewater Treatment Facility (Lowell, MA) every morning before each experiment and transported immediately to the laboratory. In the laboratory, the mixed liquor was mixed, aerated and its mixed liquor suspended solids (MLSS) was measured according to standard methods [25]. As part of the requirements for the respiration inhibition test conducted later, the mixed liquor was concentrated to a MLSS of 2000 mg/L based on the initial MLSS information [26].

Half of the concentrated mixed liquor was sheared to release the EPS from the activated sludge flocs and the other half was left as unsheared. A commercial Waring blender (model 5011) was used to successfully shear the mixed liquor for 5 min on high (22,000 rpm) [23]. The blender was wrapped with ice packs to prevent temperature increases during the shearing process.

2.3. EPS content quantification

Both the sheared and unsheared samples were filtered through a 1.0 μm glass fiber filter by using a 25 mL syringe (and stored at −20 °C if not analyzed immediately). The EPS content was quantified through soluble protein and carbohydrate analyses to determine the release of EPS as a result of mechanical shearing. A Total Protein Kit (Micro-Lowry Peterson's Modification) was obtained from Sigma–Aldrich [27], BSA was used as standard. The Dubois method was used for carbohydrate analysis [28], dextrose was used as standard. A UV–visible spectrophotometer (Agilent 8453) was used to measure the absorbance. DNA concentrations were also measured using a fluorometer (Hoefer DyNA Quant 200, Amersham Biosciences) and calf thymus as a standard solution of known DNA concentration [29]. DNA concentrations were measured to test for any disruption in cell viability during the shearing process. The COD was measured as well to indirectly examine the biological activity [25].

2.4. Activated sludge respiration inhibition test

Following the shearing, a six-beaker jar tester (Phibbs & Birds P700) was used to conduct respiration inhibition tests. A series of samples were prepared by using the pre-concentrated activated sludge, synthetic feed, a reference substance (3,5-dichlorophenol) or MWCNTs and distilled water (see Table 1). Each reactor had a final concentration of 500 mL. During the respiration inhibition test, each reactor was continually mixed at 90 rpm and aerated with a Pasteur pipette at 1 L/min for 3 h. At the end of the 3-h contact time, the contents of each reactor were placed in a 300 mL BOD

Table 1
Reactor preparation in the respiration inhibition test.

	2000 mg/L Activated sludge (mL)	Feed (mL)	DI (mL)	Volume used (mL) (final concentration)
MWCNTs	200	16	204	80 (0.64 g/L) 80 (1.44 g/L) 80 (2.16 g/L) 80 (3.24 g/L)
3.5-Dichlorophenol (0.5 g/L)	200	16	279 (5 mg/L) 274 (10 mg/L) 259 (25 mg/L)	5 (5 mg/L) 10 (10 mg/L) 25 (25 mg/L)
Controls 1 and 2	200	16	284	0

bottle and DO readings (YSI Model 52CE and probe) were taken in 1 min interval for about 10 min. The respiration rates in mg O_2/L/h were determined through the slope of the linear portion of the DO vs. time curve. All respiration rates best-fit lines had regression coefficients (R^2) greater than 0.98. See Fig. 1 for the experimental setup.

Toxicity in the form of respiration inhibition was determined by performing a Respiration Inhibition Test according to [26]:

Synthetic sewage feed: The composition of the synthetic sewage feed includes: 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. The chemicals were mixed in 1 L of distilled water and diluted 100 times before input into the batch reactors.

Reference substance/controls: 3,5-Dichlorophenol was used as a reference substance of known inhibition at concentrations of 5, 10, and 25 mg/L. An effective-concentration of 50% inhibition (EC50) of 3,5-dichlorophenol should be between 5 and 30 mg/L. This range is used to verify the sensitivity of the activated sludge. Controls (with no MWCNTs or reference) were prepared for both unsheared and sheared mixed liquor with only mixed liquor and synthetic sewage feed. Control respiration rates were acquired at the beginning and end of the experimental period (C1 and C2) to monitor changes in the physiological characteristics of the sludge with time. These values were in the desired range of each other as outlined by the EPA guidelines (15% of each other).

Test substance (R_s): The test substance (i.e., MWCNTs) of four concentrations was put into both unsheared and sheared mixed liquor combined with synthetic sewage feed. Each concentration was run in duplicate.

Respiration rates: Respiration rates were determined at the end of the 3-h contact time. The percent inhibition was obtained accord-

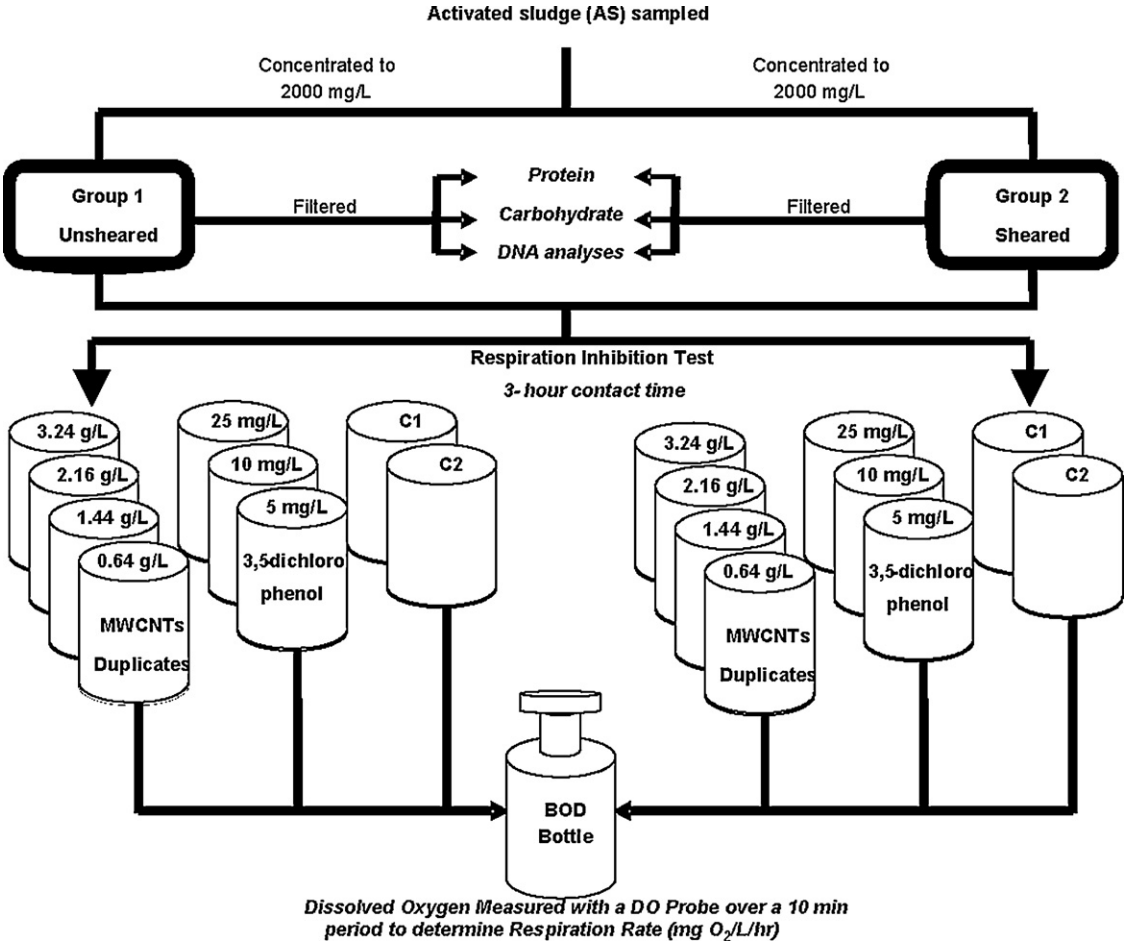

Fig. 1. Experimental schematic.

ing to Eq. (1) (against the control average):

$$\%\,\text{Inhibition} = \left(1 - \frac{R_s}{((C_1 + C_2)/2)}\right) \times 100\% \quad (1)$$

where R_s is respiration rate from MWCNTs (test substance), mg/L/h; C_1 is control 1 respiration rate, acquired from the beginning of the test, mg/L/h; C_2 is control 2 respiration rate, acquired at the end of the test, mg/L/h.

All experiments were performed under room temperature ($22 \pm 2\,^{\circ}C$) and at a pH range of 6–8.

Statistical analysis was conducted using Student's *t*-tests with two-tailed distribution. The significance level used to evaluate statistical significance was $\alpha = 0.05$.

2.5. SEM preparation

MWCNTs in distilled water: MWCNTs at a high concentration of 3.24 g/L were dispersed in distilled water according to Section 2.1. The sample was prepared by mounting to a silicon wafer and imaged by using a Field Emission Scanning Electron Microscope (JSM 7401F).

MWCNTs in activated sludge: MWCNTs at a high concentration of 3.24 g/L in the unsheared mixed liquor and sheared mixed liquor were prepared for SEM imaging by using a Field Emission Scanning Electron Microscope (JSM 7401F). The samples were taken immediately upon input of MWCNTs into the mixed liquor and at the end of the 3-h contact time before the respiration rates were obtained. One milliliter samples were collected and mounted to a polycarbonate membrane filter (24 mm, pore size 0.4 μm; Electron Microscopy Sciences) and fixed in 0.1 M phosphate buffer (pH 7.3) containing 2.5% glutaraldehyde for 2 h at 4 °C. After fixation, samples were rinsed three times in 0.1 M phosphate buffer (pH 7.3; 10 min/wash) and dehydrated gradually after successive immersions in ethanol solutions of increasing concentration (50, 70, 80, 90, 100%) (10-min/dehydration step) [30]. Drying was completed by incubating the samples for 2 h at 30 C. All samples were gold-coated before imaging to reduce charging of the sample.

2.6. Particle induced X-ray emission (PIXE)

The impurities of the MWCNTs used were independently checked by Elemental Analysis, Inc. (Lexington, KY) with a method called PIXE. This method conducts a 72-element scan for the elements of sodium through uranium at ppm detection limits. A 200 mg powdered CNT sample was prepared by palletizing a 1-in. dish between two Kapton films. Each palletized sample was placed in a plastic snap together holder located in the sample carousel. The carousel positions each sample for irradiation. The PIXE system is composed of a General Ironex 4 MV tandem accelerator with a duoplasmatron source, a dual quadrapole focusing lense, an *x*–*y* beam scanner, a beam pulser with 50 ns response time and a vaccum/helium chamber. Results from the PIXE analysis were used to verify the CNT composition information provided by Sigma–Aldrich.

3. Results and discussion

3.1. Shearing the mixed liquor: release of EPS

The concentrations of the soluble protein and carbohydrate were dramatically increased upon shearing the mixed liquor (see Table 2); the protein went from 0.29 to 67.9 mg/L and carbohydrate went from 8.14 to 26.4 mg/L, suggesting that EPS from the floc matrix was released into the bulk liquid. After shearing, protein (67.9 mg/L) was the most dominant biopolymer released compared to those values measured for the carbohydrate (8.14 mg/L); this finding is consistent with other studies [23].

Table 2
Concentration of carbohydrate, protein, and DNA of the unsheared and sheared mixed liquor.

	Unsheared (mg/L)	Sheared (mg/L)	*t*-test *P*
Carbohydrate	8.14 ± 0.65	25.98 ± 2.77	0.0004[a]
Protein	0.26 ± 0.11	67.93 ± 7.22	8.43E−05[a]
DNA	2.67 ± 1.15	3.67 ± 0.58	0.25

[a] *Note*: All concentration values are derived from triplicate samples. The difference in data is statistically significant when $P < 0.05$.

No significant increase in the DNA concentrations was observed after shearing. A significant increase in DNA concentration would be expected if cells were destroyed and their DNA was released into the bulk liquid upon shearing. These results provide evidence to support the respiration inhibition seen in this study (see Section 3.2) would not be associated with the mechanical shearing of the mixed liquor, but with the presence of MWCNTs in contact with the microbial communities present in the mixed liquor.

3.2. Respiration inhibition test data

The respiration inhibitions observed at different MWCNTs concentrations are illustrated in Table 3. The average control respiration rates were 7.36 (± 0.59) mgO_2/L/h for the unsheared mixed liquor sample and 7.69 (±0.29) mgO_2/L/h for the sheared mixed liquor sample. The control data demonstrated that shearing itself did not affect the respiration inhibition. For both unsheared and sheared mixed liquor, the respiration rates progressively decreased when the MWCNTs concentration increased, e.g. at 1.44 g/L of MWCNTs, the respiratory activity of the unsheared and sheared mixed liquor was 28 (±2)% vs. 51 (±1)%, respectively; an increase of 23% ($p = 0.006$) in respiration inhibition under the sheared conditions. The lowest respiration rate was observed when the MWCNTs concentration was the highest, demonstrating that MWCNTs imposed toxicity in the form of respiration inhibition for both unsheared and sheared mixed liquor.

Statistical analysis showed that the differences in the respiration inhibition were only significant for two MWCNT concentrations studied, 1.44 and 2.16 g/L. Although the differences seen at both 0.64 and 3.24 g/L were statistically insignificant due possibly to experimental errors, much can be derived from the two concentrations (1.44 and 2.16 g/L) where the differences were statistically significant suggesting the role of EPS could be very significant.

Most of the EPS contains a heterogeneous mixture of functional groups and possess both hydrophobic and hydrophilic properties [31]. It has been suggested that EPS can bind by hydrophobic interaction [21]. Pristine carbon nanotubes are hydrophobic in pure water [19]. Therefore, it is expected that EPS in the activated sludge flocs would bind and absorb MWCNTs to prevent MWCNTs from penetrating deeper into the heart of the flocs where most microbial communities are present and reduce their toxicity. A release of EPS from the floc matrix by mechanical shearing provided more chance for the cells to get in touch with MWCNTs, made the cell membranes of the microorganisms more readily exposed to a potential toxin (MWCNTs in this study) and subsequently lead to cell membrane damage and cell death, a mechanism suggested by Kang et al. [8,9]. Other studies have revealed possible generation of reactive oxygen species (ROS) that can damage DNA, proteins, and membranes due to the exposure of cells to nanoparticles;

Table 3
Respiration rate for both sheared and unsheared mixed liquor.

Concentration of MWCNTs (g/L)	Respiration rate (mg O_2/L/h)		Respiration inhibition (against the control average, %)		*t*-test (on respiration inhibition)
	Unsheared	Sheared	Unsheared	Sheared	*P*
Control average	7.36 ± 0.59	7.69 ± 0.29	0	0	0.56
0.64	5.88 ± 1.17	5.52 ± 0.14	20 ± 16	28 ± 2	0.55
1.44	5.28 ± 0.17	3.76 ± 0.06	28 ± 2	51 ± 1	0.006[a]
2.16	3.55 ± 0.13	3.07 ± 0.13	52 ± 2	60 ± 2	0.04[a]
3.24	2.35 + 0.03	2.27 + 0.52	68 + 1	70 + 7	0.67

[a] The difference in data is significant if $P < 0.05$.

specifically, those that are carbon-based: SWCNTs, MWCNTs, and fullerenes [12]. In addition, the sheared conditions released EPS into the solution resulting in higher soluble organic concentration in the solution, helping the MWCNTs to become less aggregated and thus more contact with bacteria. More dispersed MWCNTs will result in greater inactivation of cells [13,32]. Together, this result demonstrates the toxicity impact of MWCNTs and the protective ability of EPS to the microbial communities in the activated sludge.

3.3. SEM images

Fig. 2 shows the MWCNTs in distilled water. The image demonstrates that the MWCNTs were dispersed and distributed evenly as loose aggregates after the sonication process. It also illustrates that MWCNTs existed in this form when they were input into the activated sludge reactors. The average diameter of the MWCNTs observed was approximately 20 nm.

Three images were obtained for the mixture of MWCNTs and activated sludge flocs. Fig. 3a shows that cohesive and compact aggregates between the MWCNTs and activated sludge flocs were formed as soon as the MWCNTs were added, illustrating the interaction between the flocs and MWCNTs was immediate. At the end of the 3-h contact time, images obtained demonstrate a direct and close physical contact of rod-shaped bacteria (Fig. 3b) and small flocs (Fig. 3c) with the aggregates and fibers of the MWCNTs. These images suggest that some of the CNTs could be brought into close contact with the microorganisms within the flocs due to mechanical mixing and longer contact time.

Agglomeration and absorption of nanoparticles to the activated sludge have been reported to be significant [18]. Such interaction could subsequently increase the retention time of CNTs in the wastewater treatment process, pose chronic toxicity to the

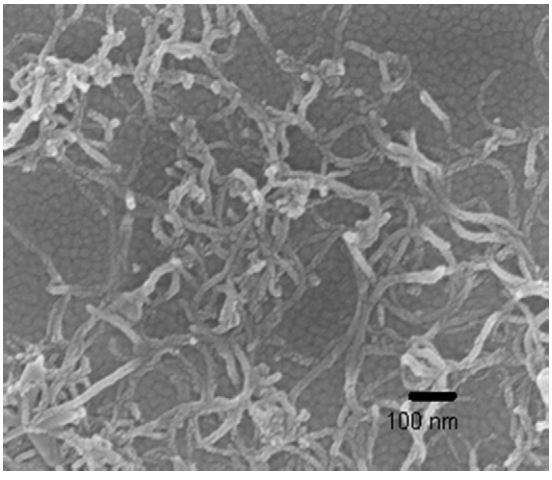

Fig. 2. SEM image of MWCNTs (3.24 g/L) dispersed in distilled water at 65,000× magnification.

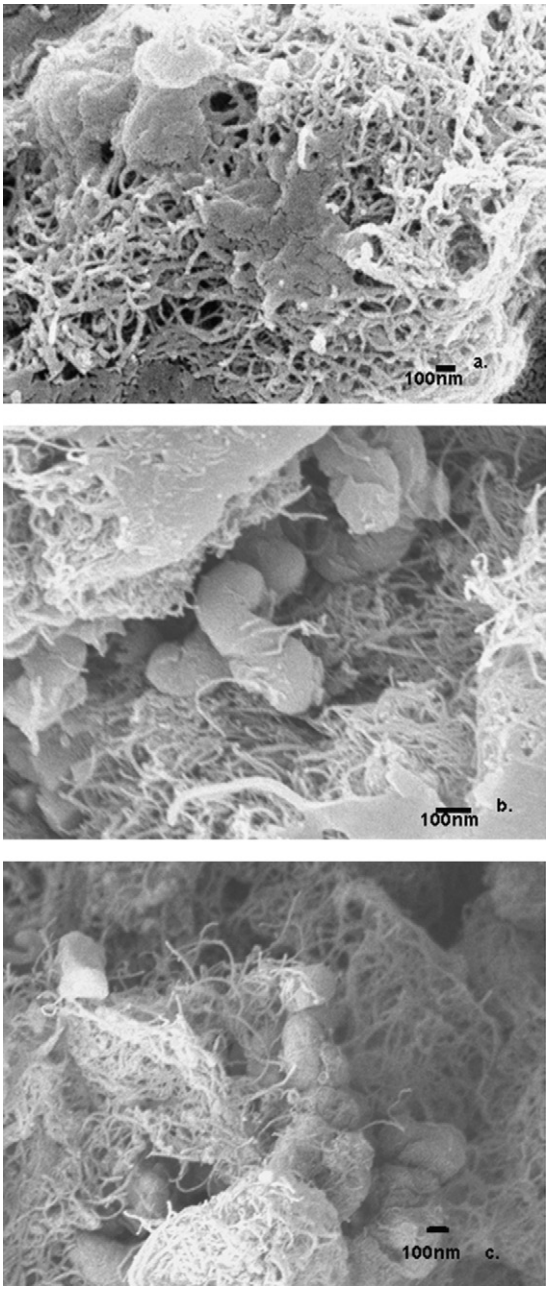

Fig. 3. SEM images of MWCNTs and activated sludge: (a) MWCNTs at 3.24 g/L immediately after input into activated sludge. (b) MWCNTs at 3.24 g/L after a 3-h contact time with unsheared activated sludge. (c) MWCNTs at 3.24 g/L after a 3-h contact time with sheared activated sludge.

microbial community, and make the removal of nanoparticles difficult.

3.4. COD data

No direct correlation was found between the respiration inhibition rates and the soluble COD removal rates (data not shown). It appears that soluble organic matter (measured by the soluble COD) was strongly absorbed by MWCNTs and such absorption increased with the concentration of MWCNTs. This observation is consistent with the work published by Yin et al. [19] who reported that 17% of the soluble COD was absorbed by 250 mg/L of SWCNTs. This result also helps to explain that the release of EPS after shearing created an environment where absorption of MWCNTs to more soluble organic matter occurred and made MWCNTs more dispersed and thus more contact with the cells. In addition, it suggests that soluble COD is not an accurate indicator of biological activity when CNTs are present due to their absorption of organic matter.

3.5. PIXE result

The PIXE analysis' results showed that there was 95.78% carbon content, 2.19% Al content, and 1.98% Fe content contained in the sample of MWCNTs used. These results are consistent with the manufacturer's reported impurity levels (see Section 2.1).

4. Conclusions

This paper used a respiration inhibition test to demonstrate the potential toxicity posed by MWCNTs on the microbial communities in the activated sludge and to illustrate the extent of EPS in protecting the microorganisms from the toxicity of CNTs. The main conclusions that can be drawn from this study are summarized as follows:

- MWCNTs imposed toxicity in the form of respiration inhibition to the microbial communities in the activated sludge. The respiration inhibition observed was determined to be dose-dependent; the highest concentration of MWCNTs exhibited the highest respiration inhibition.
- The sheared mixed liquor exhibited greater respiration inhibition when in contact with MWCNTs compared to the unsheared mixed liquor. It demonstrated that EPS played a significant role in protecting the microbial communities in the activated sludge against the toxicity posed by the MWCNTs: mechanical shearing of mixed liquor and the subsequent release of EPS into the bulk liquid allowed the microbial communities to be more readily exposed to the MWCNTs, which subsequently resulted in more respiration inhibition for the sheared mixed liquor compared to the unsheared mixed liquor.
- Direct physical contact between the microorganisms and MWCNTs was illustrated by the SEM images and these images showed close interactions between MWCNTs and activated sludge flocs.
- The concentrations of MWCNTs used for this study were high to ensure effects would be measured if there was any. Future study should focus on expanding the concentration range to include lower concentrations and other types of CNTs, e.g. SWCNTs and functionalized CNTs.

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