Ecotoxicology and Environmental Safety 88 (2013) 9–15

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# High concentrations of single-walled carbon nanotubes lower soil enzyme activity and microbial biomass

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ARTICLE INFO

*Article history:*
Received 3 July 2012
Received in revised form
27 September 2012
Accepted 10 October 2012
Available online 6 December 2012

*Keywords:*
Nanomaterial
Carbon nanotube
Soil microorganism
Extracellular enzyme
Surface area
Soil incubation

ABSTRACT

Nanomaterials such as single-walled carbon nanotubes (SWCNTs) may enter the soil environment with unknown consequences resulting from the development of nanotechnology for a variety of applications. We determined the effects of SWCNTs on soil enzyme activity and microbial biomass through a 3-week incubation of urban soils treated with different concentrations of SWCNTs ranging from 0 to 1000 μg $g^{-1}$ soil. The activities of cellobiohydrolase, β-1,4-glucosidase, β-1,4-xylosidase, β-1,4-N-acetylglucosaminidase, L-leucine aminopeptidase, and acid phosphatase and microbial biomass were measured in soils treated with powder and suspended forms of SWCNTs. SWCNTs of concentrations at 300–1000 μg $g^{-1}$ soil significantly lowered activities of most enzymes and microbial biomass. It is noteworthy that the SWCNTs showed similar effects to that of multi-walled carbon nanotubes (MWCNTs), but at a concentration approximately 5 times lower; we suggest that this is mainly due to the higher surface area of SWCNTs than that of MWCNTs. Indeed, our results show that surface area of CNTs has significant negative relationship with relative enzyme activity and biomass, which suggests that greater microorganism—CNT interactions could increase the negative effect of CNTs on microorganisms. Current work may contribute to the preparation of a regulatory guideline for the release of CNTs to the soil environment.



## 1. Introduction

Carbon nanotubes (CNTs) have been widely studied due to their excellent size-dependent physicochemical properties, large surface area to weight ratio, and superb electrical, optical, thermal, and mechanical properties for various applications (Javey et al., 2003; Han et al., 2004; Hayamizu et al., 2008; Sekitani et al., 2008; Welsher et al., 2009; Baloch et al., 2012; Kim et al., 2012). CNTs are also employed in the form of composites that can enhance the properties of conventional materials to be lighter, stronger, and more conductive (Shin et al., 2012). Owing to the extensive potential applications, CNTs could enter the environment from disposal, abrasion, and export processes, and soil environment is the ultimate recipient to which the CNTs will be accumulated (Gottschalk et al., 2009; Klaine et al., 2008; Muller and Nowack, 2008). However, studies on the potential negative impacts of CNTs on soil biota including microorganisms that play important role in nutrient cycling are still limited (Nowack and Bucheli, 2007; Chung et al., 2011; Dinesh et al., 2012; Zhao and Liu, 2012).

Research on the effects of CNTs on microorganisms have been limited mainly to culture studies, which were carried out in less complex and more controlled environment than the soil environment. Antimicrobial properties of CNTs were observed from these culture studies, and this implies that CNTs may negatively affect microorganisms in soils as well. For example, CNTs upon contact inhibited growth and biofilm maturation of bacteria (Arias and Yang, 2009; Rodrigues and Elimelech, 2010). In addition, CNTs that were dispersed using different surfactants and those in aggregates showed antimicrobial properties when incubated with bacteria (Liu et al., 2009; Bai et al., 2011). The main mechanism shown for the antimicrobial activity of CNTs was physical piercing of microbial cells rather than oxidative stress or metal residues present in the CNTs (Kang et al., 2008; Liu et al., 2009).

The effect of CNTs on microorganisms in environmental samples such as activated sludge and wastewater effluent have been determined in a few studies, and they also showed that CNTs repressed microbial activity (Luongo and Zhang, 2010; Goyal et al., 2010; Kang et al., 2009). For instance, the addition of CNTs inhibited the respiration and altered the structure of the microbial communities residing in activated sludge (Goyal et al., 2010; Luongo and Zhang, 2010). CNTs also inactivated a large portion of bacteria in rivers and wastewater effluent (Kang et al., 2009). When CNTs enter soils, soil organic matter (SOM) could mitigate their effect on microorganisms because the mobility of CNTs can

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http://dx.doi.org/10.1016/j.ecoenv.2012.10.031

be dampened when adsorbed to SOM (Dinesh et al., 2012). On the contrary, SOM may increase the mobility and bioavailability of CNTs by acting as natural surfactants (Navarro et al., 2008). These potentially complicated interactions between microorganisms and soil matrix, and the observation that the toxicity of CNTs on bacterial cultures was not an accurate predictor of microbial inactivation in environmental samples (Kang et al., 2009) call for studies on the effect of CNTs in soils.

On the other hand, recent culture studies suggest that SWCNTs have a higher antimicrobial activity than MWCNTs because they have smaller diameter and thus a larger surface area, which leads to greater interaction with microbial cells (Kang et al., 2008; Arias and Yang, 2009). When the effect of SWCNTs and MWCNTs were compared, SWCNTs exhibited significantly higher cytotoxicity and induced a higher concentration of DNA and RNA efflux from microbial cells than MWCNTs (Kang et al., 2008; Arias and Yang, 2009). These studies suggest that SWCNTs exhibit higher microbial cytotoxicity than MWCNTs, and can exert negative influence on microorganisms at a lower concentration (Kang et al., 2008). However, since the effect of SWCNTs on soil microbial activity has not been reported so far, direct comparison between SWCNTs and MWCNTs in terms of their effects on soil microorganisms has not been possible.

We investigated the effects of SWCNTs on enzyme activity and microbial biomass in soils treated with SWCNTs. Extracellular enzymes play an important role in terrestrial ecosystems because they catalyze rate-limiting steps in decomposition and nutrient cycling (Sinsabaugh, 1994). Moreover, soil microbial enzyme activity and biomass are sensitive indicators of changes in microbial communities under soil disturbance such as contamination by MWCNTs and heavy metal (Khan and Scullion, 2000; Chaperon and Sauvé, 2008; Chung et al., 2011). We extended our previous study on the effect of MWCNTs on soil microbial activity and tested our hypothesis that SWCNTs will have negative effect on soil microorganisms at a lower concentration than MWCNTs due to a higher surface area. For this, SWCNTs of a broad range of concentrations (from 30 to 1000 μg, SWCNTs $g^{-1}$ soil) were added to soils, and the highest concentration is 5 times lower than the concentration of MWCNTs at which soil microbial activity and biomass were significantly repressed (Chung et al., 2011). In addition, two different forms of SWCNTs that have distinct relevance to the environmental exposure were applied to soils. We demonstrate for the first time in our knowledge that application of SWCNTs at high concentrations can significantly lower soil microbial enzyme activity and biomass, and that there is a significant negative relationship between surface area of CNTs and relative enzyme activities.

## 2. Materials and methods

### 2.1. Soil sampling

Surface soil (upper 15 cm) was collected in July 2011 from a landscaped site dominated by grasses on Korea University campus. This site was chosen because it is a representative urbanized area in Korea and the effect of MWCNTs on soil microorganisms at this site has been previously studied by our group (Chung et al., 2011). Soil samples were sieved through an 8 mm sieve after being collected from the field. The texture of soils was sandy loam, and the pH was $6.98 \pm 0.20$. The soil C and N concentrations were $17.69 \pm 0.22$ g C $kg^{-1}$ soil and $1.14 \pm 0.03$ g N $kg^{-1}$ soil, respectively. Cation exchange capacity (CEC) was $13.51 \pm 0.78$ cmol CEC $kg^{-1}$ soil (Chung et al., 2011).

### 2.2. Preparation and characterization of SWCNTs

SWCNTs (Southwest Nanotechnologies, Inc., USA) were obtained by a gas-phase catalytic method. According to the information provided by the supplier, SWCNTs were grown with a supported cobalt–molybdenum (Co–Mo) bimetallic catalyst on silica particles using a fluidized bed reactor by disproportionation of carbon monoxide. The purification of SWCNTs was performed through oxidation of the Co and Mo catalysts and removal of the catalysts and the silica support (Buffa et al., 2005). SWCNTs used in our work have an average length of 1.02 μm and average diameter of 1.0 nm with an aspect ratio of 1000. The result of thermogravimetric analysis showed $>90$ wt% of carbon content. We determined specific surface area of SWCNTs by the Brunauer, Emmet, and Teller (BET) method (Brunauer et al., 1938) using ASAP 2010 (Micromeritics Inc., USA), and it was 1125.3 $m^2 g^{-1}$. The surface area of MWCNTs was 237.1 $m^2 g^{-1}$, and other detailed information on MWCNTs are described elsewhere (Chung et al., 2011).

The experiment was implemented using powder and suspended forms of SWCNTs. 'Powder form' SWCNTs refer to SWCNTs that were not treated in any way, i.e., they are identical to the form received from the purchaser. We chose 'powder form' SWCNTs because this is the form that microorganisms will encounter when there is an accidental spill from manufacturing facilities of CNTs. On the other hand, SWCNTs are often suspended in solution when being applied during fabrication process of various products (Someya, 2009; Welsher et al., 2009). Therefore, we also tested the effect of 'suspension form' of SWCNTs which was prepared by bath-sonicating the mixture of SWCNTs and deionized (DI) water at room temperature for 5 min.

### 2.3. Soil incubation

Eighty grams of soil subsamples were placed in short-form straight jars ($n=4$). Powder and suspended forms of CNTs were added to soils and mixed. The treatments included control (DI water only), 30, 100, 300, 600 and 1000 μg SWCNT $g^{-1}$ soil. Soil samples were preincubated at 22 °C for 1 week before adding SWCNTs to allow time for microbial activity to stabilize. The soils were incubated at 22 °C and the soil moisture content was adjusted to initial weight by adding DI water regularly.

### 2.4. Extracellular enzyme assays

The activity of six extracellular enzymes involved in soil C, N, and P cycling was measured. Acid phosphatase cleaves phosphoester bonds, whereas β-1,4-glucosidase and cellobiohydrolase degrade cellulose. β-1,4-xylosidase decomposes hemicelluloses, and β-1,4-N-acetylglucosaminidase degrades chitin. L-leucine aminopeptidase is a protein-degrading enzyme. Extracellular enzyme activities were determined by fluorogenic substrate methods following Saiya-Cork et al. (2002) and DeForest (2009). Briefly, soil slurries were made with 2 g of soil samples and 125 ml sodium acetate buffer. These slurries were then homogenized and added to the black 96-well microplate. Substrates and standards were added and enzyme activities were determined using the Multilabel Plate Reader (Perkin–Elmer Inc., USA). These assays were performed at 2 h, 3 days, 7 days, 15 days, and 23 days after the application of SWCNTs. The enzyme activities are expressed as nmol 4-MUB $g^{-1} h^{-1}$.

### 2.5. Microbial biomass C and N analyses

Soil microbial biomass was determined 32 days after the SWCNTs were applied to soils using the chloroform fumigation–extraction method according to Vance et al. (1987). Five grams of soils were fumigated for 3 days with chloroform, extracted with 0.5 M $K_2SO_4$, and analyzed for total organic carbon and total organic nitrogen contents using a TOC-VCPH/CPN analyzer (Shimadzu, Japan). Microbial biomass is expressed as μg C $g^{-1}$ soil or μg N $g^{-1}$ soil.

### 2.6. Statistical analyses

All statistical analyses were conducted by SAS version 9.3 (SAS Inst. Inc., Cary, NC, USA). We used two-way analyses of variance to determine the effect of SWCNTs on enzyme activity and microbial biomass. Significant effects of SWCNT treatment, incubation time, and their interactions were accepted at $\alpha=0.05$. To determine which means differ from other means within a group, Tukey's honestly significant difference test was employed ($P<0.05$).

In addition, linear regression analyses were performed to determine the relationship between surface area of diverse forms of CNTs and microbial parameters. For this purpose, we compiled relative enzyme activity and microbial biomass data obtained from this study and those extracted from Chung et al. (2011)'s study, and analyzed if surface area of CNTs that have different forms and wall numbers has a significant relationship with microbial characteristics. Relative enzyme activity is the percentage of enzyme activity under each different concentration of CNTs over enzyme activity of the control treatment. Likewise, relative microbial biomass is the percentage of microbial biomass under each different CNT concentration over microbial biomass under control treatment. Relative enzyme activity and relative microbial biomass were calculated using data from 11th day of incubation in soils treated with MWCNTs (Chung et al., 2011) and those from 15th day of incubation acquired from this study. Data of these two time points were chosen because 11th day was the last time point at which the enzyme activities were determined in Chung et al. (2011)'s study, and 15th day in current study is the closest time point to this. Data on L-leucine aminopeptidase were obtained only from current study, and thus were not included in regression analysis. Surface area of CNTs per gram soil was derived by multiplying specific surface area of CNTs by the concentration of CNTs applied to soils.