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Responses of soil ammonia-oxidizing microorganisms to repeated exposure of single-walled and multi-walled carbon nanotubes

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HIGHLIGHTS

- The impact of repeated exposure of carbon nanotubes on the microbial communities was investigated.
- The first exposure caused stronger effects on microbial biomass compared with the second exposure.
- SWNTs and MWNTs produced different effects on the net N nitrification.
- The abundance and diversity of AOA were higher than that of AOB under the exposure of carbon nanotubes.

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ABSTRACT

The impacts of carbon nanotubes (CNTs) including single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) on soil microbial biomass and microbial community composition (especially on ammonium oxidizing microorganisms) have been evaluated. The first exposure of CNTs lowered the microbial biomass immediately, but the values recovered to the level of the control at the end of the experiment despite the repeated addition of CNTs. The abundance and diversity of ammonium-oxidizing archaea (AOA) were higher than that of ammonium-oxidizing bacteria (AOB) under the exposure of CNTs. The addition of CNTs decreased Shannon–Wiener diversity index of AOB and AOA. Two-way ANOVA analysis showed that CNTs had significant effects on the abundance and diversity of AOB and AOA. Dominant terminal restriction fragments (TRFs) of AOB exhibited a positive relationship with NH_4^+, while AOA was on the contrary. It implied that AOB prefer for high-NH_4^+ soils whereas AOA is favored in low NH_4^+ soils in the CNT-contaminated soil.

1. Introduction

Nanotechnology has provided a basis for innovation in a wide range of fields and resulted in an exponential increase in products of novel materials (Gao et al., 2013; Maurer-Jones et al., 2013). Carbon nanotubes (CNTs) are one of the most promising nanomaterials and have become the subject of numerous investigations in chemical, physical and material science research areas since its discovery in 1991 (Iijima, 1991; Odom et al., 1998). Based on their outstanding physicochemical and mechanical properties, CNTs could be potentially applied in consumer electronics, building materials, agricultural smart delivery and so on (Rodrigues and Elimelech, 2010; Tong et al., 2012; Li et al., 2013a). CNTs can be classified as single-walled carbon nanotubes (SWNTs), which are hollow tubes of carbon capped at either end with a hemi-fullerene (Iijima and Ichihashi, 1993), and multi-walled carbon nanotubes (MWNTs) consisting of concentric layers of graphene sheets, where smaller diameter tubes are encased in larger diameter tubes (Ebbesen et al., 1993).

With their expanding role of CNTs in manufacturing, some environmental exposure is raising concerns about their potential environmental risk (Helland et al., 2007). The knowledge on the impacts of CNTs on the environment microbes is currently limited and most researches have been limited to pure culture studies (Ahmed et al., 2012; Rodrigues and Elimelech, 2010), which were carried out in less complex and more controlled environment than in the soil environment (Jin et al., 2013). Toxicity studies taking *Escherichia coli* (*E. coli*) as a model microorganism showed that over 80% of the cells attached to non-functionalized SWNTs aggregates died after an incubation of 60 min (Kang et al., 2008). Gene analysis of the *E. coli* which was exposed to CNTs showed an efficient expression of stress-related genes, corroborating the cytotoxic effects of CNTs on *E. coli* (Rodrigues and Elimelech, 2010). There are a handful of studies focused on the

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toxic effects of CNTs on microbial communities (Rodrigues et al., 2013). However, the majority of the studies addressed the bacteria in activated sludge (Goyal et al., 2010; Luongo and Zhang, 2010), wastewater (Kang et al., 2009), animals (Lam et al., 2004; Roberts et al., 2007; Zhang et al., 2008) and plants (Caňas-Carrell et al., 2008; Stampoulis et al., 2009). Although some recent studies have addressed the fate of CNTs in soil, few studies have investigated their toxicity on microbial communities in soil environment (Li et al., 2013b, 2013c). Jin et al. found that SWNTs can reduce the soil enzyme activity and alter the microbial community structures (Jin et al., 2013, 2014). Shrestha et al. observed that there was no effect of MWNTs on soil respiration, enzymatic activities, and microbial community at 10, 100 and 1000 mg kg^{-1} soil (Shrestha et al., 2013), while a study indicated that most enzyme activity was repressed under MWNTs of 1000 mg kg^{-1} soil (Chung et al., 2011). These different results are divergent. Furthermore, the effects of repeated exposure of CNTs on the soil microorganisms are still not clear.

Nitrification is a key process in the soil nitrogen (N) cycle (Tan et al., 2013). Ammonium-oxidizing microorganisms (AOM) including AOB (Rousidou et al., 2013) and AOA (Konneke et al., 2005) have been identified as the key group that controls the rate-limiting step of nitrification i.e. the oxidation of ammonium to hydroxylamine (Prosser and Nicol, 2008). A previous study reported that the abundance of AOB and AOA could serve as a relevant and cost-effective bio-indicator for soil monitoring (Wessén and Hallin, 2011). Meanwhile, Pereira-Silva et al. suggested that ammonium-oxidation represents a well measurable disturbance-sensitive microbial process and proposed that analysis of the abundance and diversity of AOM could provide a good estimation of soil health (Pereira-Silva et al., 2013). Studies on the pollutants that affect AOM have been mostly focused on fertilization (Zhou et al., 2014), pesticides (Li et al., 2008; Wan et al., 2014), and nitrification inhibitor (Kleineidam et al., 2011). To our knowledge, the effects of nanomaterials on AOM, especially CNTs, have still not been explored.

In this study, the impacts of repeated exposure of SWNTs or MWNTs on soil microbial activity were investigated. The potential effects on AOB and AOA were examined using quantitative real-time PCR (qPCR) analysis and terminal restriction fragments length polymorphism (T-RFLP) during a short term laboratory incubation experiment. The results will provide a wide range of data to characterize the communities of AOM and stimulate a subsequent research aiming at the risk assessment of CNTs that were repeatedly accumulated in soil environment.

2. Materials and methods

2.1. CNTs characterization

Commercial CNTs were purchased from Cheng du Organic Chemicals Co. Ltd., Chinese Academy of Sciences. CNTs were characterized before they were applied. To characterize the shape and size distribution of CNTs, samples were prepared by depositing liquid samples on TEM grid and air drying and analyzed using a high resolution transmission electron microscope (HRTEM) (JEM-2100F STEM). The thermal stability of the CNTs was determined using thermo-gravimetric analysis (TGA) by heating the CNTs from room temperature to 1100 °C at a rate of 10 °C/min under nitrogen atmosphere (Pyris Diamond TG/DTA, Perkin Elmer instruments). Specific surface area of CNTs was measured by the Brunauer–Emmet–Teller (BET) method (Quantachrome, Quantasorb SI). The information of the characteristics of the CNTs was presented in the appendices.

2.2. Soil sample collection and physicochemical characterization

Soil was collected from the upper 10 cm of cropland used for planting wheat in the northwest of Jinan, Shandong province, China (36°38′ 51″ N, 116°51′ 11″ E). The soil was fully mixed and sieved through a 2 mm sieve to remove the plant material and large particles for incubation. The soil is a sandy loam (sand 70.52%, silt 20.27%, and clay 9.21%). The main physicochemical characteristics were determined by standard methods (Li, 1983) and are listed as follows: bulk density 1.36 g cm^{-3}, pH 7.24, water content 8.58%, organic matter 2.19%, NO_3^-–N 7.25 mg kg^{-1}, and NH_4^+–N 2.09 mg kg^{-1}.

2.3. Soil exposure to CNTs

The CNTs suspension was ultrasonicated in deionized water for three 30-min cycles. The detailed information was referred by Rodrigues et al. (2013). Before incubation, the soil was adjusted to 40% maximal water holding capacity (WHC_{max}) and preincubated in the dark at 25 ± 1 °C for 7 days. At the end of the preincubation, the soil samples were treated with CNTs. Suspended CNTs were added to 600 g of soil subsamples placed in trays and thoroughly mixed with a glass rod. The concentrations of SWNTs applied to soils were 100 (S1), 200 (S2), and 500 μg g^{-1} soil (S3). The concentrations of MWNTs were 100 (M1), 500 (M2), and 1000 μg g^{-1} soil (M3). Control without any CNTs was treated with distilled water (CK). Each treatment in triplicates was adjusted to 40% WHC_{max} and incubated at 25 ± 1 °C.

The treatments consisted of two successive doses of CNTs at the same concentration on day 0, and day 7. During the incubation, deionized water was added every day to compensate for any water lost. Soil was collected and determined with the biochemical and microbial properties at four time phases (day 0, 3, 7, and 14) from each tray.

2.4. Microbial biomass carbon and inorganic nitrogen assays

Microbial biomass carbon (C) was determined by the fumigation extraction method (Vance et al., 1987). Organic C content of the K_2SO_4-extracts was measured using the titration method. Microbial biomass C was calculated with $K_{EC} = 0.38$ and expressed as μg C per gram dry soil. Ammonium concentration (NH_4^+–N) and nitrate concentration (NO_3^-–N) were determined colorimetrically according to Bao (2005).

The rate of net N nitrification (Rnit) was computed according to the following equation (Chen et al., 2014).

$$\Delta t = t_{i+1} - t_i \quad (1)$$

where i is the last analyzed time and i + 1 is the present analyzed time.

$$Rnit = \left[(NO_3^- - N)_{j+1} - (NO_3^- - N)_j\right]/\Delta t. \quad (2)$$

2.5. Molecular analyses

2.5.1. Quantitative real-time PCR (qPCR) analysis

DNA was extracted using the Powersoil DNA isolation kit (MOBIO) according to the manufacturer's instructions. The abundance of AOB, AOA and bacteria was determined by quantitative polymerase chain reaction (qPCR) using the primers amoA1-F/amoA2-R (Tan et al., 2013), Arch-amoAF/Arch-amoAR (Strauss et al., 2014), and 16Sf/16Sr (Wan et al., 2014), respectively. The qPCR reaction (20 μl) contained 10 μl of SYBR® Select Master Mix (2×), 0.6 μl of each primer, 1 μl of template DNA, and 7.8 μl double-distilled water. Real-time PCR was conducted using an ABI7500 (Applied Biosystem, USA). Each PCR run started with a hot start at 50 °C for 2 min and 95 °C for 10 min, and then continued with 40 thermo-cycling 95 °C for 15 s, 60 °C for 60 s and 72 °C for 30 s. The copy numbers were determined via standard curve constructed as follows: Ten-fold serial dilutions of a known copy number of the plasmid DNA were subjected to a real-time PCR assay in triplicate to generate an external standard curve.