Influence of multi-walled carbon nanotubes on the microbial biomass, enzyme activity, and bacterial community structure in 2,4-dichlorophenol-contaminated sediment

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Abstract

The rise in manufacture and use of carbon nanotubes has aroused the concern about their potential risks associated with coexisting pollutants in the aquatic environment. 2,4-dichlorophenol (2,4-DCP), with a high toxicity to many aquatic organisms, is a widespread pollutant resulting from the extensive use of pesticides and preservatives. In this article, the adsorption of 2,4-DCP by riverine sediment and the responses of sediment microbial community to 2,4-DCP were studied in the presence of multi-walled carbon nanotubes (MWCNTs). Adding MWCNTs significantly increased the adsorption amount of sediment for 2,4-DCP from 0.541 to 1.44 mg/g as the MWCNT concentration increased from 0 to 15 mg/g. The responses of sediment microbial community were determined after one-month exposure to MWCNTs at different concentrations (0.05, 0.5, 5, and 50 mg/g). The microbial biomass carbon in the sediment contaminated with 2,4-DCP increased in the presence of 5 mg/g of MWCNTs (from 0.06 to 0.11 mg/g), but not significantly changed at other MWCNT concentrations. For the sediments contaminated with 2,4-DCP, the presence of MWCNTs made no difference to urease activity, while the dehydrogenase activity slightly increased with the addition of 5 mg/g of MWCNTs and decreased in the presence of 50 mg/g of MWCNTs. The changes of sediment bacterial communities were further determined by 16S rRNA gene sequencing. Based on the weighted UniFrac distance between communities, the clustering analysis suggested that the contamination of 2,4-DCP affected the bacterial community structure in a greater degree than that caused by MWCNTs at relatively low concentrations (≤ 5 mg/g).

Bacteroidetes, *Planctomycetes*, and *Nitrospirae* were feature bacterial phyla to reflect the effects of MWCNTs and 2,4-DCP on sediment bacterial community. These results may contribute to the understanding of microbial community response to co-exposure of MWCNTs and 2,4-DCP and the assessment of associated ecological risks.

Keywords: Carbon nanotubes; Microbial community; Sediment; 2,4-dichlorophenol; 16S rRNA gene

1. Introduction

As a kind of one-dimensional nanomaterials with many attractive properties, carbon nanotubes (CNTs) have been widely studied and applied in various fields, such as composites, microelectronics, medicine, energy, and environment (Schnorr and Swager 2011, Song et al. 2018a). Currently, global production capacity of CNTs has reached thousands of tons per year, and the global market is $3.95 billion in 2017 and expected to rise to $9.84 billion by 2023 (Tamez Ramírez and Vega-Cantú 2019). According to the layer number of tube walls, CNTs are divided into single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs). MWCNTs are preferred in industrial applications due to their lower cost, which makes them the majority of current CNT market (Zhai et al. 2016, Bishop et al. 2017). Mass manufacture and use of CNTs will certainly result in their release into the environment, as CNTs can enter into the environment during the whole product life cycle including manufacturing, processing, service stage, recycling, and disposal (Köhler et al. 2008). Most of the released CNTs will eventually enter into the natural waters and accumulate in sediments (Liu and Cohen 2014). The predicted concentrations of CNTs in Europe soil, sludge treated soil, and sediment were 1.51, 73.6, and 241 ng/kg/y, respectively (Gottschalk et al. 2009). According to the simulation by Koelmans et al. (2009), the total concentration of manufactured carbon-based nanoparticles in aquatic sediment was 1.2–2000 μg/kg, and the CNT concentration might reach a higher level in the future. This has aroused concern about the potential risks of CNTs in the aquatic environment.

Due to the nanoscale radial dimension and unique microstructure, CNTs can cause toxic effects on fish, invertebrates, algae, and microorganisms in the aquatic environment (Chen et al. 2018). Additionally, CNTs may interact with coexisting pollutants and change their mobility, toxicity, and bioavailability, especially for hydrophobic organic pollutants with aromatic rings (Myer et al. 2017, Song et al. 2017a, Fan et al. 2018, Song et al. 2018b). This kind of pollutants can be strongly adsorbed by CNTs via hydrophobic interaction and π-π interaction between the aromatic rings of organic compounds and the highly polarizable graphene sheets of CNTs (Jesionowski et al. 2014). Our previous study showed that incorporating 0.5%, 1.0%, and 1.5% (w/w) MWCNTs into riverine sediment significantly increased the adsorption capacity of sediment for sodium dodecyl benzene sulfonate and impeded its transport through the sediment column (Song et al. 2018b). Fan et al. (2018) found that 50 mg/L of MWCNTs could decrease the toxicity and bioavailability of paraquat (0.82 mg/L) to *Arabidopsis thaliana*, and the protective effect on root surface area against paraquat toxicity was attributed to the adsorption of paraquat by MWCNTs. Although many valuable results have been obtained, efforts are still required to advance the understanding of environmental risks of CNTs.

Sediment microbial communities are vital to maintaining the health and services of aquatic ecosystems (Song et al. 2018b, Orland et al. 2019). Compared with fish, invertebrates, and algae, sediment microbial communities are more likely to be exposed to CNTs and pollutants in sediment as microorganisms are virtually present in all sediment environments. The interactions among CNTs, pollutants, and

microbial communities should be studied for a better assessment of the ecological risks of CNTs (Xie et al. 2016, Song et al. 2017b). As an important industrial material, 2,4-dichlorophenol (2,4-DCP) is extensively used for manufacturing pesticides (e.g., 2,4-dichlorphenoxyacetic acid, oxadiazon, and nitrofen), medicines (e.g., bithionol), and preservatives (e.g., triclosan). The occurrence of 2,4-DCP in aquatic environment mainly arises from the widespread use of pesticides and the wastewater discharge from related industrial activities (Zhou et al. 2017). In the surface water of China, the concentrations of 2,4-DCP were reported ranging from < 1.1 to 19,960 ng/L (Gao et al. 2008). Exposure to 2,4-DCP can cause pernicious effects to many aquatic organisms, such as oxidative damage to *Daphnia magna* (Wu et al. 2011), growth inhibition to algae (Ertürk and Saçan 2012), and DNA damage in fish (Huang et al. 2018), and even cause human health risks via food and drinking water (Igbinosa et al. 2013). It is significant to understand the ecological risks of 2,4-DCP. To our knowledge, there are few studies about the interactions among CNTs, 2,4-DCP, and microbial communities in sediment. In this research, MWCNTs were added to 2,4-DCP-contaminated sediment at the concentrations of 0.05, 0.5, 5, and 50 mg/g, and microbial community in the sediment was analyzed after one-month exposure. These MWCNT concentrations were chosen to match those used in previous related studies (Chung et al. 2011, Kerfahi et al. 2015), and the extremely high concentration of MWCNTs (50 mg/g) was considered a worst case of accidental spills or CNT waste accumulation (Shrestha et al. 2013). The main purpose of this study is to assess the impacts of MWCNTs on the microbial biomass

carbon, enzyme activity, and bacterial community structure in 2,4-DCP-contaminated riverine sediment. It is expected that this study would promote the understanding of ecological impacts of CNTs and associated risks.

2. Materials and methods

2.1. Sediment and MWCNTs

Sediment was collected from Changsha section of the Xiangjiang River, the largest river in Hunan Province of China. Prior to use, the sediment was air-dried, crushed, ground, sieved to less than one mm, and manually homogenized. The contaminated sediment was prepared by spiking 2,4-DCP with the following procedures. Firstly, 2,4-DCP was first dissolved in CH_2Cl_2 and mixed with 25% (by weight) of the total sediment. For evaporating the solvent completely, the mixture was stirred every 15 min. Then, the treated sediment was mixed with the rest 75% of sediment. After manual homogenization, 50% (v/w) ultrapure water was added to adjust the moisture content of spiked sediment. The final concentration of 2,4-DCP in the spiked sediment was 30 mg/kg. The 2,4-DCP concentration for treatment was decided at this level based on the consideration of both the environmental concentration of chlorophenol and the experimental concentrations used in previous related studies (Khairy 2013, Zhou et al. 2013, Dallinger and Horn 2014, Zhou et al. 2017). MWCNTs were bought from Chengdu Organic Chemistry Co., China. According to the technical data provided by the manufacturer, the CNT content, length, and outer diameter of MWCNTs are > 90%, 5–20 μm, and 10–20 nm,

respectively. There are some impurities of Fe and O in the MWCNTs, and the C content is > 95%. The microstructures of MWCNTs and sediment were characterized by scanning electron microscope (SEM, Fig. S1). The determined pH value and organic carbon content of sediment were 7.92 and 1.63% (w/w), respectively. Sediment elemental composition was analyzed by energy disperse spectroscopy (EDS), and O, Si, Al, Fe, C, and K were the main component elements (Fig. S1e).

2.2. Adsorption experiments

The adsorption experiments were performed in conical flasks containing 2,4-DCP solutions of various concentrations (from 10–80 mg/L), using MWCNTs, sediment, and sediment-MWCNT mixtures as adsorbents. The 2,4-DCP solutions were made through diluting the stock solution of 2,4-DCP dissolved in methanol. For minimizing the cosolvent effect, methanol concentrations in the prepared 2,4-DCP solutions were controlled below 0.1% (v/v) (Wang et al. 2014). The adsorbent dosage of MWCNTs was 0.3 g/L, and the dosages of sediment and sediment-MWCNT mixtures were both 20 g/L. The MWCNT concentration in the sediment-MWCNT mixtures was 0.05, 0.5, 5, and 50 mg/g, respectively. These conical flasks were shaken at 180 rpm and 25 ± 1 °C for 24 h, followed by static settlement of the adsorbents for 6 h. The concentrations of 2,4-DCP in the supernatant were measured by high performance liquid chromatography (HPLC, Agilent 1100, USA) equipped with a reversed-phase C18 column and a variable wavelength detector. Methanol solution (70%, v/v) was used as the mobile phase with a flow rate of 1 mL/min. The

detection wavelength was 284 nm.

2.3. Experimental design for MWCNT exposure

Ten treatments in triplicate were implemented to investigate the impacts of MWCNTs on microbial community in riverine sediment contaminated with 2,4-DCP (detailed experimental design is displayed in Table S1 of the Supplementary Material). MWCNT powders were added to the uncontaminated and contaminated sediments at the concentrations of 0 (T1 and T2), 0.05 (T3 and T4), 0.5 (T5 and T6), 5 (T7 and T8), and 50 mg/g (T9 and T10), respectively. The MWCNT-sediment mixtures were manually homogenized. The sediment moisture was kept at 50% (v/w) and adjusted daily. A month later, samples were taken out for the subsequent tests.

2.4. Measurement of microbial biomass and enzyme activity

Microbial biomass carbon was measured by fumigation-extraction method (Deng et al. 2016). Specifically, 12.5 g of sediment were fumigated with ethanol-free chloroform at 25 °C for 24 h to lyse microbial cells and release cellular content. After removing the chloroform, the sediment was transferred to a conical flask and 50 mL of 0.5 M K_2SO_4 extractant were added. The mixture was shaken for 30 min before centrifugal treatment. Subsequently, the supernatant was filtered and the total organic carbon in filtrate was determined by a total organic carbon analyzer. Another 12.5 g of sediment without fumigation were used as the control, following the same extraction procedure.

178 Urease activity was quantified by the amount of NH_4^+ produced from
179 urease-mediated urea hydrolysis within a certain reaction time, and the concentration
180 of NH_4^+ was measured by the indophenol blue colorimetric method (Liu et al. 2018).
181 Specifically, 0.5 mL toluene was added to 1 g of sediment in a centrifuge tube. After
182 15 min, 2.5 mL of 10% urea solution and 5 mL of citrate buffer solution (pH 6.7)
183 were added. The sample tube was then incubated at 37 °C for 24 h. After that, the
184 mixture was centrifuged, and the supernatant was collected and diluted 10 times with
185 ultrapure water. Subsequently, 4 mL of the diluted supernatant was moved to another
186 centrifuge tube, and 0.8 mL of 1.35 M sodium phenate solution and 0.6 mL of sodium
187 hypochlorite solution (active chlorine 0.9%) were added. After 20 min, the mixture
188 was diluted with ultrapure water to a final volume of 10 mL and the absorbance at 578
189 nm was recorded. The same procedures without urea substrate (using ultrapure water
190 instead of urea solution) were conducted on another 1 g of sediment as the control.
191 Dehydrogenase activity was measured by a reduction reaction using
192 2,3,5-triphenyl tetrazolium chloride (TTC) as the hydrogen acceptor, and quantified
193 by the amount of generated red 2,3,5-triphenyl formazan (TF) by colorimetric method
194 (Mierzwa-Hersztek et al. 2016). Specifically, 2 mL of 1% TTC solution, 2 mL of
195 Tris-HCl buffer solution (pH 7.6), and 2 mL of ultrapure water were added to 1.5 g of
196 sediment. Then, the mixture was incubated at 37 °C for 6 h, and the formed TF was
197 extracted by 5 mL of methanol. After filtration, the absorbance at 485 nm was
198 measured. The same procedures without the addition of TTC (using ultrapure water
199 instead of TTC solution) were performed on another 1.5 g of sediment as the control.

2.5. DNA extraction and 16S rRNA gene sequencing

Microbial genomic DNA was extracted from 200 mg of sediment using the Mag-Bind® Environmental DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the protocol provided by the manufacturer. The integrity of extracted DNA was checked by agarose gel electrophoresis. Subsequently, the genomic DNA was accurately quantified for polymerase chain reaction (PCR) with the Qubit™ ssDNA Assay Kit in a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The hypervariable V3-V4 region of bacterial 16S rRNA genes was amplified by nested PCR with the primer 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Zhang et al. 2018). The PCR products were separated by agarose gel electrophoresis, and the DNA was purified and recovered with *MagicPure*™ DNA Size Selection beads (TransGen Biotech Co., Beijing, China). The recovered DNA was accurately quantified prior to the 16S rRNA gene sequencing, and 20 pmol samples were used for the sequencing by an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

2.6. Data analysis

The results of adsorption experiments were fitted by Langmuir and Freundlich isotherm models to analyze the effects of MWCNTs on the adsorption of 2,4-DCP by sediment (detailed description of the two models is shown in the Supplementary Material).

Microbial biomass carbon (mg/g) was calculated with the following equation:

$$\text{Microbial biomass C} = \frac{(C_{f+} - C_{f-}) \times V}{0.45 \times m} \quad (1)$$

where C_{f+} (mg/L) and C_{f-} (mg/L) are the concentrations of total organic carbon measured from the sediments with and without fumigation, V (L) is the extract volume, m (g) is the sediment weight, and 0.45 is a conversion factor (Deng et al. 2016). The difference value calculation of total organic carbon can eliminate the possible influence of MWCNTs on the measurement of microbial biomass carbon. Urease activity (μg NH_4^+/g/h) was calculated according to the following equation:

$$\text{Urease activity} = \frac{(C_{u+} - C_{u-}) \times V_3 \times V_1 \times n}{T \times m \times V_2} \quad (2)$$

where C_{u+} (μg/L) and C_{u-} (μg/L) are the concentrations of NH_4^+ measured with and without urea substrate, V_1 (L) is the total extract volume, V_2 (L) is the volume of diluted supernatant for colorimetric test, V_3 (L) is the final volume of colored solution, m (g) is the sediment weight, n is the dilution ratio, and T (h) is the incubation time. Dehydrogenase activity (μg TF/g/h) was calculated with the following equation:

$$\text{Dehydrogenase activity} = \frac{(C_{T+} - C_{T-}) \times V}{T \times m} \quad (3)$$

where C_{T+} (μg/L) and C_{T-} (μg/L) are the concentrations of TF measured with and without the addition of TTC, V (L) is the extract volume, m (g) is the sediment weight, and T (h) is the incubation time. Analysis of variance (ANOVA) was carried out to determine the differences between group mean values, and a P-value less than 0.05 suggests a statistically significant difference.

Sequenced reads were clustered into different operation taxonomic units (OTUs) at a similarity level of 97%. The corresponding taxonomic information was obtained

using the Ribosomal Database Project Classifier (Wang et al. 2007).

The β-diversity differences between different treatments were quantified using the unique fraction metric (UniFrac) (Lozupone et al. 2011). The weighted UniFrac distance that takes into account the difference in proportional abundance of the taxa between two samples was calculated based on a phylogenetic tree generated by FastTree using maximum likelihood method (Price et al. 2010). The values of weighted UniFrac distance range from zero to one, and a smaller distance indicates a more similar phylogenetic structure between two communities.

Linear discriminant analysis (LDA) effect size (LEfSe) method was used to determine the feature taxa most likely to explain the differences between different treatments. LEfSe couples the factorial Kruskal-Wallis test for statistical significance with the pairwise Wilcoxon test for biological consistency, and the discriminative features are ranked by effect size based on LDA (Segata et al. 2011). In the LEfSe analysis of this study, the α values for the factorial Kruskal-Wallis test among classes and the pairwise Wilcoxon test between subclasses were both 0.05, and the non-negative threshold on the logarithmic LDA score for discriminative features was 3.5.

3. Results and discussion

3.1. Effects of MWCNTs on the adsorption of 2,4-DCP by sediment

The presence of MWCNTs in sediment significantly affected the adsorption of

266 2,4-DCP by sediment. Measured and fitted isotherms of 2,4-DCP adsorption by
267 MWCNTs, sediment, and sediment-MWCNT mixtures are illustrated in Fig. 1. Fitting
268 parameters of the Langmuir model and the Freundlich model are displayed in Table 1.
269 Both the two models showed a good fitting effect to the results with a minimum
270 R^2-value of 0.944. According to the estimated values of q_m and K_F, there were about
271 two orders of magnitude difference between MWCNTs and sediment in the adsorption
272 amount for 2,4-DCP. The maximum adsorption amount of MWCNTs was 47.6 mg/g,
273 while that of sediment was only 0.541 mg/g. This result can be explained by the
274 strong interactions between MWCNTs and 2,4-DCP. On the one hand, the
275 hydrophobic nature of both MWCNTs and 2,4-DCP made them easily aggregated in
276 aqueous solution through hydrophobic interaction (Kragulj et al. 2015). On the other
277 hand, the π-π stacking between the benzene ring of 2,4-DCP and the graphene sheets
278 of MWCNTs further enhanced the adsorption (Apul and Karanfil 2015).

279 After the incorporation of MWCNTs, the adsorption capacity of sediment for
280 2,4-DCP significantly increased (P = 0.003). Compared with the pure sediment, the
281 sediment with 5, 10, and 15 mg/g of MWCNTs showed a 0.85-fold, 1.18-fold, and
282 1.66-fold increase in the q_m value, respectively (Table 1). This phenomenon generally
283 occurs when CNTs show high adsorption capacity for pollutant while the interaction
284 between sediment and pollutant is relatively weak. Based on the adsorption
285 experiments, biota-sediment accumulation factor (BSAF) for 2,4-DCP in sediments
286 with different concentration of MWCNTs was estimated (Table S2). BSAF is widely
287 used for assessing the risk of pollutant accumulation by biotas from sediment (Shen et

al. 2018). The estimated BSAF decreased from 31.8 to 6.6 with the increasing MWCNT concentration in sediment. This result indicates a lower bioavailability and biodegradation of 2,4-DCP in sediment in the presence of MWCNTs. In a previously reported study, Zhou et al. (2013) found that CNTs could inhibit the 2,4-DCP degradation and mineralization in soil, as the adsorption of 2,4-DCP by CNTs decreased the bioavailability of 2,4-DCP. Additionally, CNTs and 2,4-DCP might produce combined effects on the sediment microorganisms. Therefore, the effects of MWCNTs on microbial biomass, enzyme activity, and bacterial community structure in the sediment contaminated with 2,4-DCP were further studied.

3.2. Effects of MWCNTs on the microbial biomass and enzyme activity

The changes in microbial biomass carbon of the sediments from different treatments are presented in Fig. 2a. For the sediments uncontaminated with 2,4-DCP, the microbial biomass carbon significantly decreased from 0.23 to 0.18 and 0.09 mg/g in the presence of 5 and 50 mg/g of MWCNTs, respectively. This result was consistent with previously reported findings that high concentrations of MWCNTs (≥ 5 mg/g) could cause an obvious decrease in the microbial biomass carbon of soil (Chung et al. 2011, Shrestha et al. 2013). The antibacterial property of MWCNTs could be accounted for this phenomenon. When a large number of MWCNTs were added into the sediment, microbes might be wrapped by long MWCNTs, which resulted in the isolation of microbes from the external environment and their growth inhibition (Smith and Rodrigues 2015). Additionally, the sharp ends of short

310 MWCNTs might pierce the cell membrane, and lead to severe damage to the microbes
311 (Song et al. 2018a). The toxicity of 2,4-DCP decreased the microbial biomass carbon
312 in a condition of the same MWCNT concentration. Compared with the sediment
313 contaminated with only 2,4-DCP (T2), the addition of 0.05, 0.5, and 50 mg/g of
314 MWCNTs had no significant effects on the microbial biomass carbon, while that
315 increased from 0.06 to 0.11 mg/g in the presence of 5 mg/g of MWCNTs. The
316 increased microbial biomass carbon might result from the decreased toxicity of
317 2,4-DCP due to the adsorption by MWCNTs, but the effect was lost with higher
318 concentration of MWCNTs as a result of the significant antibacterial effect caused by
319 a large number of MWCNTs (Song et al. 2019).

320 Urease is an important extracellular enzyme for nitrogen mineralization and it is
321 widely used for assessing the nutrient cycling and microbial nutrient demand
322 (Cordero et al. 2019). The sediment urease activity in different treatments is
323 illustrated in Fig. 2b. The sediments uncontaminated with 2,4-DCP exhibited higher
324 urease activity than that of contaminated sediments, which indicates the inhibiting
325 effect of 2,4-DCP to the sediment urease. According to a study by Bello et al. (2013),
326 soil urease activity comes from two main sources: the enzymes produced by active
327 organisms, and the enzymes stabilized by soil colloids. Therefore, in this study, the
328 decreased urease activity might be because the toxic effect of 2,4-DCP on microbes
329 inhibited the activity of urease produced by active organisms. Besides, no significant
330 difference in the urease activity was found between the sediments contaminated with
331 2,4-DCP (T2, T4, T6, T8, and T10), suggesting the relative stability of urease

332 stabilized by sediment colloids. For the sediments uncontaminated with 2,4-DCP,
333 adding 5 mg/g of MWCNTs increased the urease activity from 5.0 to 6.6 μg NH_4^+/g/h,
334 while no significant differences were found between the other treatments. The
335 stabilizing effect of 5 mg/g of MWCNTs for the free urease released by active
336 organisms could be the cause, as the adverse effect of MWCNTs at that concentration
337 on microbes might not be very large and MWCNTs could stabilize the urease via
338 physical adsorption (Feng and Ji 2011, Jesionowski et al. 2014).

339 Dehydrogenase meaningfully participates in pollutant degradation, organic
340 matter transformation, and microbial metabolism, which enables it to be widely used
341 for studying the changes of microbial oxidative activity in contaminated sites (Tan et
342 al. 2017b). The sediments contaminated with 2,4-DCP showed a slightly higher
343 dehydrogenase activity than that of uncontaminated sediments, but the differences
344 were not significant. Compared with the sediments without MWCNTs (T1 and T2),
345 the dehydrogenase activity of sediment with 5 mg/g of MWCNTs slightly increased,
346 while the dehydrogenase activity slightly decreased in the presence of 50 mg/g of
347 MWCNTs. A significant difference in dehydrogenase activity was found between the
348 sediments containing 5 and 50 mg/g of MWCNTs. The increased dehydrogenase
349 activity might result from higher metabolic activity due to higher energy consumption
350 of microbes to resist the MWCNT stress (Tan et al. 2017a), while the evident
351 antibacterial effect of 50 mg/g of MWCNTs decreased the dehydrogenase activity
352 (Chung et al. 2011). These results indicate that MWCNTs could have a direct impact
353 on microbes and thus affect the enzymes they produce. Though the important roles of

354 microbial community in aquatic ecosystems are well acknowledged and understood,
355 the risk assessment of MWCNTs about their effects on the sediment microbial
356 community is lacking. The changes of microbial biomass carbon and enzyme activity
357 may provide valuable information about the potential impact of MWCNTs on the
358 whole ecosystem, especially under the stress of both MWCNTs and coexisting
359 pollutants. A two-way ANOVA was further conducted to determine the main effects of
360 MWCNTs and 2,4-DCP and their interactive effects (Table S3). The results reveal that
361 both the main effects of MWCNTs and 2,4-DCP and their interactive effects on the
362 microbial biomass carbon were significant ($P \leq 0.001$). No significant main effect of
363 MWCNTs on the urease activity ($P = 0.248$) was found, while there was significant
364 main effect of 2,4-DCP on the urease activity ($P < 0.001$). For the dehydrogenase
365 activity, significant main effect of MWCNTs ($P = 0.020$) was found. The interaction
366 between MWCNTs and 2,4-DCP showed no significant effect on neither the urease
367 activity ($P = 0.148$) nor the dehydrogenase activity ($P = 0.933$). However, the
368 microbial community structure may not change in a consistent manner with the
369 enzyme activity (ecological function), as four different outcomes are possible in
370 microbial community studies: (1) both structure and function change; (2) neither
371 structure nor function is affected; (3) only structure is altered; and (4) only function
372 varies (Frossard et al. 2012). Therefore, the bacterial community structure in
373 sediments of different treatments was further analyzed.
374

3.3. Effects of MWCNTs on the bacterial community structure

The bacterial community structure at phylum level is shown in Fig. 3a. *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were the dominant species in sediments, and they accounted for 69%–88% of the total bacterial abundance. High relative abundance of *Proteobacteria* was observed for most treatments, and its maximum proportion (59%) was found in the sediment with 50 mg/g of MWCNTs (T10). In the sediment uncontaminated with 2,4-DCP (T1), the relative abundance of *Bacteroidetes* (36%) was much higher than that of *Firmicutes* (4%), while an opposite situation in the relative abundance of these two species was found in the sediment contaminated with 2,4-DCP (T2). The incorporation of MWCNTs affected the relative abundance of bacterial community structure, and it became similar between the sediments uncontaminated and contaminated with 2,4-DCP when the MWCNT concentration was extremely high (T9 and T10). A more detailed classification of the community at class level is illustrated in Fig. 3b. *Sphingobacteriia* and *Clostridia* were the main classes of *Bacteroidetes* and *Firmicutes*, respectively. *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* are common bacterial species in the natural sediments (Zhu et al. 2013). *Proteobacteria* is the largest bacterial phylum with the most diverse phenotypes, and usually predominates in freshwater sediment (Cheng et al. 2014). *Bacteroidetes* and *Firmicutes* are more widely studied in gut microbiota, and *Firmicutes*/*Bacteroidetes* ratio has been used as an indicator of obesity due to its high correlation with body mass index and the competitive interaction between the two species (Koliada et al. 2017). In this study, obvious competitive relationship between

Bacteroidetes and *Firmicutes* was also observed, and *Firmicutes* was more dominant than *Bacteroidetes* in T2, T4, T9, and T10. The following reasons might account for this. On the one hand, many species of *Firmicutes* can participate in the anaerobic dechlorinating processes of chlorinated organic compounds and utilize them to proliferate (Zhang et al. 2015), but the activity was restricted in the presence of 0.5 and 5 mg/g of MWCNTs as the bioavailability of 2,4-DCP decreased due to the adsorption by MWCNTs. On the other hand, *Firmicutes* can form endospores against various environmental stresses (e.g., lack of nutrients, and toxicity of pollutants) while *Bacteroidetes* cannot, which makes *Bacteroidetes* more vulnerable to the pollutant toxicity (Fajardo et al. 2019). Additionally, the alpha diversity (including the Chao1 index and the Shannon index) of different sediment samples was analyzed to show the bacterial richness and diversity (Table S4). The 2,4-DCP-contaminated sediments showed higher species diversity, which might be due to the greater environmental heterogeneity (Curd et al. 2018).

The bacterial community differences between various treatments were quantified using weighted UniFrac distance, which has been widely used for describing the diversity differences between communities from different habitats (Lozupone et al. 2011). Based on the weighted UniFrac distance, all treatments were clustered into three groups (Cluster I–III in Fig. 4). Each of these groups has a distinct sediment characteristic: uncontaminated with 2,4-DCP (Cluster I), contaminated with 2,4-DCP (Cluster II), and containing MWCNTs at extremely high concentration (Cluster III). The high value of weighted UniFrac distance between Cluster I and II showed that the

contamination of 2,4-DCP affected the bacterial community structure and the impact was greater than that caused by 0.05, 0.5, and 5 mg/g of MWCNTs. The bacterial community structure in sediments uncontaminated and contaminated with 2,4-DCP became similar in the presence of 50 mg/g of MWCNTs (T9 and T10) and these two treatments were grouped into a separate cluster, indicating that the bacterial community was mainly influenced by MWCNTs rather than 2,4-DCP in that case. Additionally, the dissimilarity of bacterial community structure within Cluster II (a maximum weighted UniFrac distance of 0.392) was greater than that within Cluster I (a maximum weighted UniFrac distance of 0.241), which suggests that adding 0.5 or 5 mg/g of MWCNTs made a greater difference to the bacterial community in sediments contaminated with 2,4-DCP than that of uncontaminated sediments. This might be because MWCNTs at those concentration levels effectively adsorbed 2,4-DCP and reduced its toxicity and bioavailability to bacteria.

LEfSe is a widely used tool for high-dimensional biomarker discovery and explanation, and LEfSe algorithm was performed to further identify feature taxa in the three clusters (Segata et al. 2011). In this study, this tool was used to find feature bacteria in each cluster and determine their contributions to the differences between groups. The contributions were indicated by the logarithmic LDA score of significant different taxa (Fig. S2). The phylum *Bacteroidetes*, the genus *Sporacetigenium*, and the genus *Symbiobacterium* were feature bacterial taxa with the highest logarithmic LDA score in Cluster I, II, and III, respectively. However, these three taxa were not at the same classification level. Therefore, a cladogram that showed all significantly

different taxa at different classification level was plotted and displayed in Fig. 5. It was found that *Bacteroidetes* (log LDA score = 5.27), *Planctomycetes* (log LDA score = 4.13), and *Nitrospirae* (log LDA score = 3.93) were feature bacterial phyla in Cluster I, II, and III, respectively. Additionally, the corresponding branches showed significantly different taxa from genus to phylum level, which indicates the importance of these three phyla in distinguishing the differences between groups. Relative abundance of the three feature phyla in different groups was illustrated in Fig. S3. As a common species in the natural sediments, *Bacteroidetes* showed relatively high abundance in Cluster I, and were more vulnerable to the toxicity of 2,4-DCP and high concentration of MWCNTs, which makes them a sensitive biological indicator (Wolińska et al. 2017). *Planctomycetes* are a distinct group of bacteria that are widespread in the aquatic environment, and may be potential candidates for sediment quality assessment as this bacterial species show different dose-response behaviour to different pollutants (Flores et al. 2014). *Nitrospirae* play important roles in biological nitrification and nitrogen cycle, and similar result of the increase in relative abundance of *Nitrospirae* after CNT exposure was also reported in a previous study (Qian et al. 2018), which supports *Nitrospirae* as a feature bacterial phylum in sediment containing high concentration of CNTs. The significant differences in relative abundance of these bacterial phyla make them potential candidates to reflect the effects of MWCNTs and 2,4-DCP on sediment bacterial community.

4. Conclusions

In summary, the experimental results showed that MWCNTs could affect the potential risks of 2,4-DCP in riverine sediment. The presence of MWCNTs increased the adsorption of 2,4-DCP by sediment, thus influencing the microbial biomass carbon, enzyme activity, and bacterial community structure in sediment, especially at a MWCNT level of 5 mg/g. When the MWCNT concentration in sediment was extremely high (50 mg/g), the MWCNTs themselves had a greater impact on sediment microbial community. *Bacteroidetes*, *Planctomycetes*, and *Nitrospirae* were feature bacterial phyla to reflect the effects of MWCNTs and 2,4-DCP on sediment bacterial community. The response of microbial community may be a valuable indicator for the risk assessment of MWCNTs and coexisting pollutants in the aquatic environment. Additionally, these results could be considered when developing secure CNT applications and implementing sediment management.

Acknowledgements

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Table 1

Parameters of the isotherm models for 2,4-DCP adsorption.

Concentration of MWCNTs	Langmuir model			Freundlich model		
in sediment (mg/g)	q_m (mg/g)	K_L (L/mg)	R^2	K_F (mg/g·(mg/L)$^{-1/n}$)	$1/n$	R^2
0	0.541	0.00897	0.986	0.00918	0.737	0.980
5	1.00	0.0108	0.978	0.0227	0.696	0.988
10	1.18	0.0193	0.970	0.0546	0.601	0.994
15	1.44	0.0310	0.989	0.106	0.540	0.997
Pure MWCNTs	47.6	0.163	0.944	15.5	0.261	0.997

Figure 1

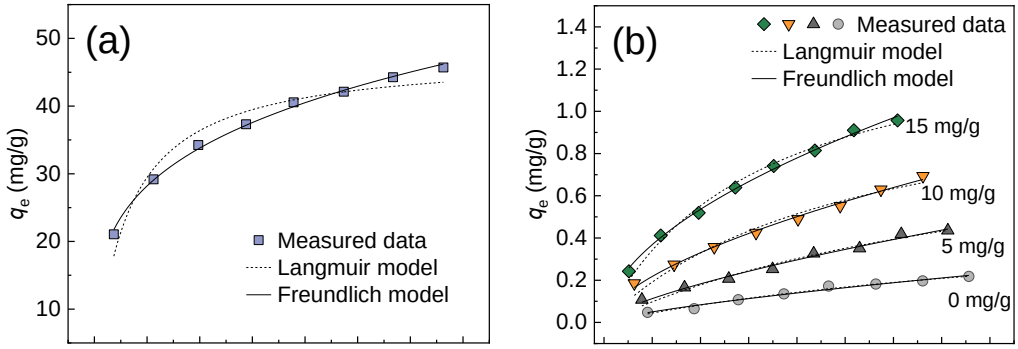

Fig. 1. Isotherms of 2,4-DCP adsorption by MWCNTs (a) and sediments with different MWCNT concentrations (b).

Figure 2

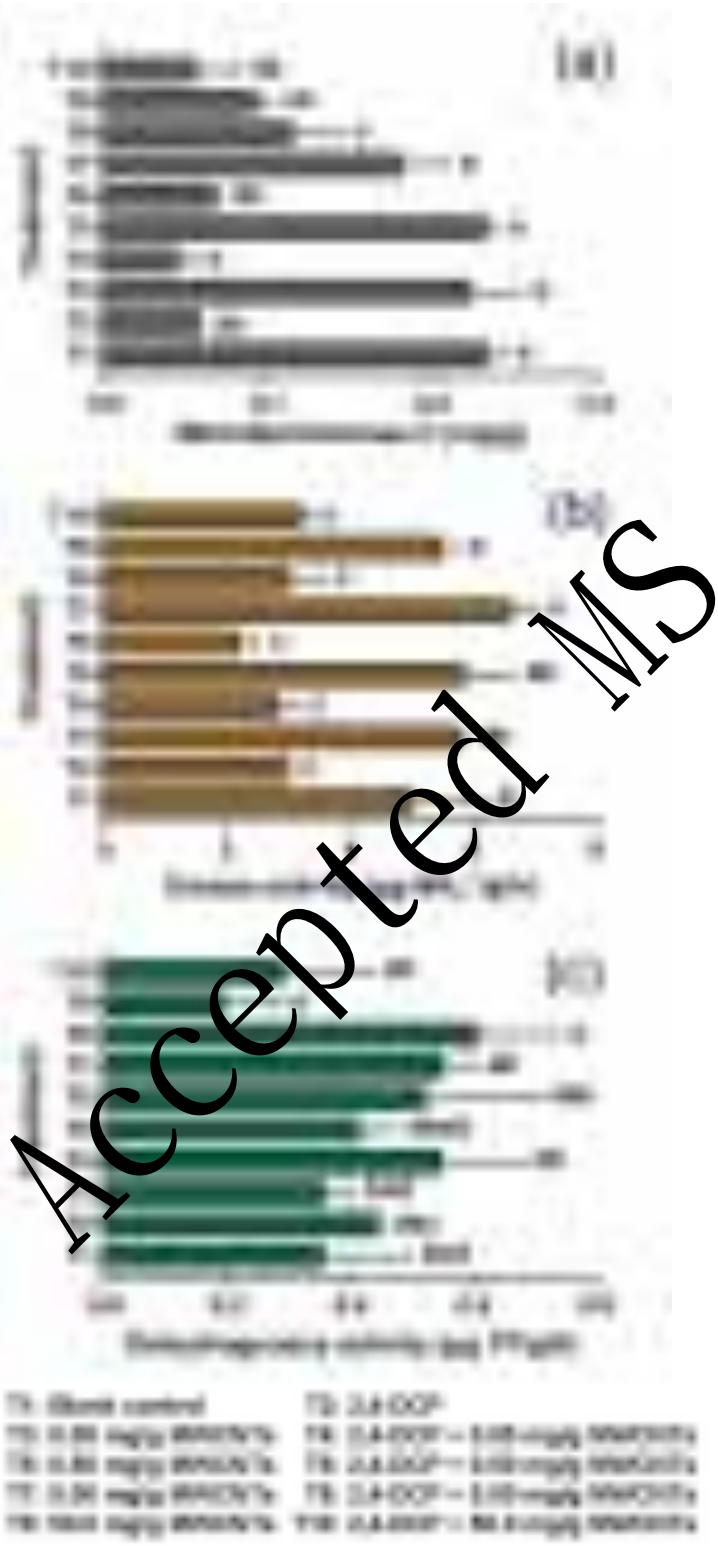

Fig. 2. Microbial biomass carbon (a), urease activity (b), and dehydrogenase activity (c) of the sediments from different treatments. Different letters denote statistically significant differences ($P < 0.05$) between groups.

Figure 3

Fig. 3. Bacterial community structure in sediments from different treatments at phylum (a) and class (b) level.

Figure 4

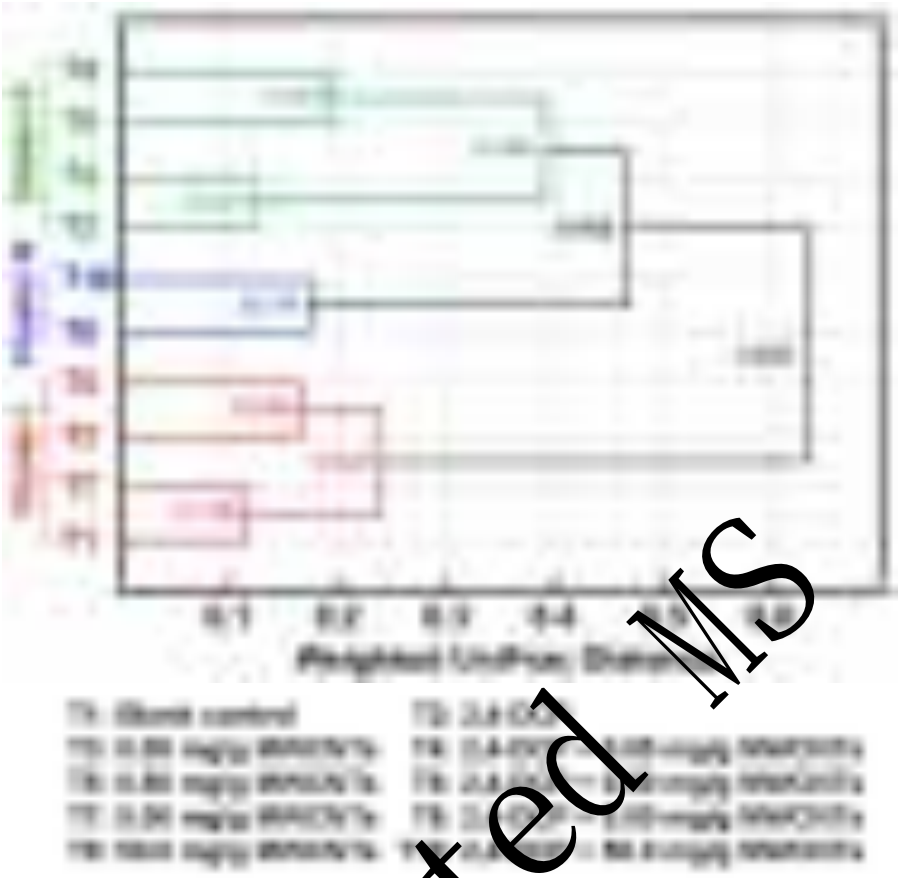

Fig. 4. Phylogenetic analysis of the bacterial communities in different treatments based on weighted UniFrac distance.

Figure 5

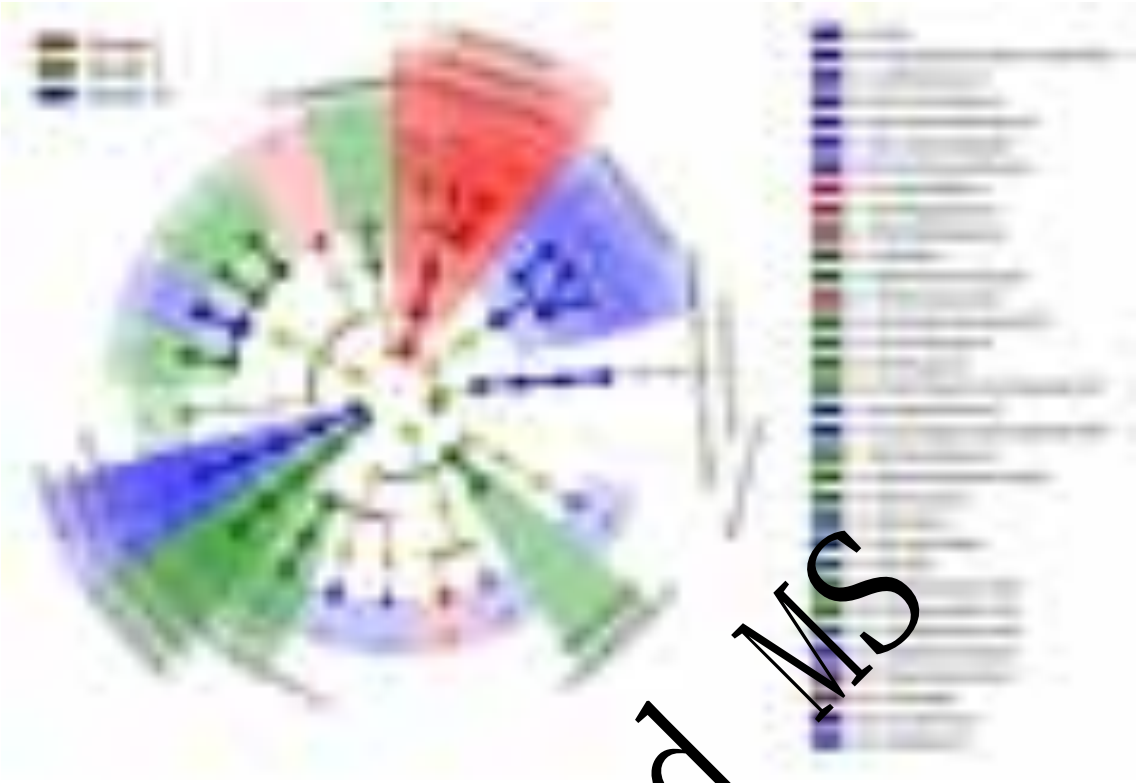

Fig. 5. Cladogram plotted from LEfSe analysis showing the significant differences ($P < 0.05$) in relative abundance of bacterial taxon among different clusters based on the analysis of weighted UniFrac distance. Different colors (red, green, and blue) indicate different clusters. The colored dots from inner to outer represent phylum, class, order, family, and genus levels, and the dot size suggests the relative abundance of bacterial taxon. The taxon with significant difference in relative abundance among clusters is shown by the dot colored in red, green, or blue, while yellow dot represents that the relative abundance of corresponding taxon is not significantly different among different clusters. The colored shadows indicate the trends of significantly different taxa. The names of significant different taxa at family and genus levels are listed on the right. Only taxa with a logarithmic LDA score more than 3.5 are shown on the cladogram.

Electronic Supplementary Material for

Influence of multi-walled carbon nanotubes on the microbial biomass, enzyme activity, and bacterial community structure in 2,4-dichlorophenol-contaminated sediment

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Calculation of adsorption amount

The adsorption amount q_e (mg/g) is calculated by the following equation:

$$q_e = \frac{(c_0 - c_e) \times V}{m}$$

where c_0 (mg/L) is the initial concentration of 2,4-DCP, c_e (mg/L) is the equilibrium concentration of 2,4-DCP, V (L) is the volume of solution, and m (g) is the weight of adsorbent.

Langmuir and Freundlich isotherm models

The Langmuir and Freundlich isotherm models are given by the following equations:

Langmuir model: $q_e = \frac{q_m K_L c_e}{1 + K_L c_e}$

Freundlich model: $q_e = K_F c_e^{\frac{1}{n}}$

where q_e (mg/g) is the amount of adsorbed 2,4-DCP at equilibrium, q_m (mg/g) is the maximum adsorption capacity, K_L (L/mg) is the Langmuir constant, c_e (mg/L) is the equilibrium concentration of 2,4-DCP, K_F (mg/g(mg/L)$^{-1/n}$) and n (dimensionless) are Freundlich constants.

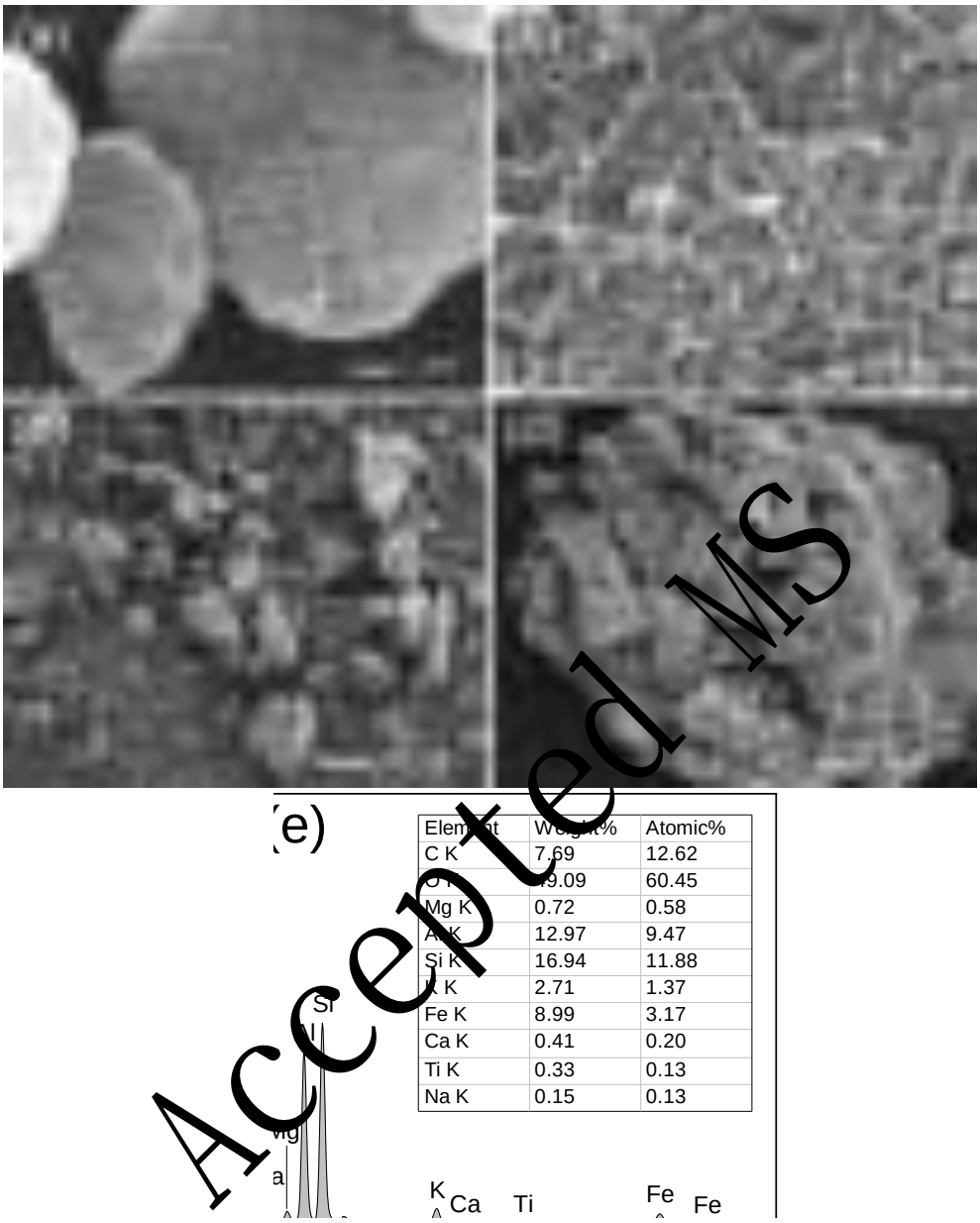

Fig. S1. SEM images of the used MWCNTs (a and b) ans sediment (c and d), and EDS analysis of the sediment (e).

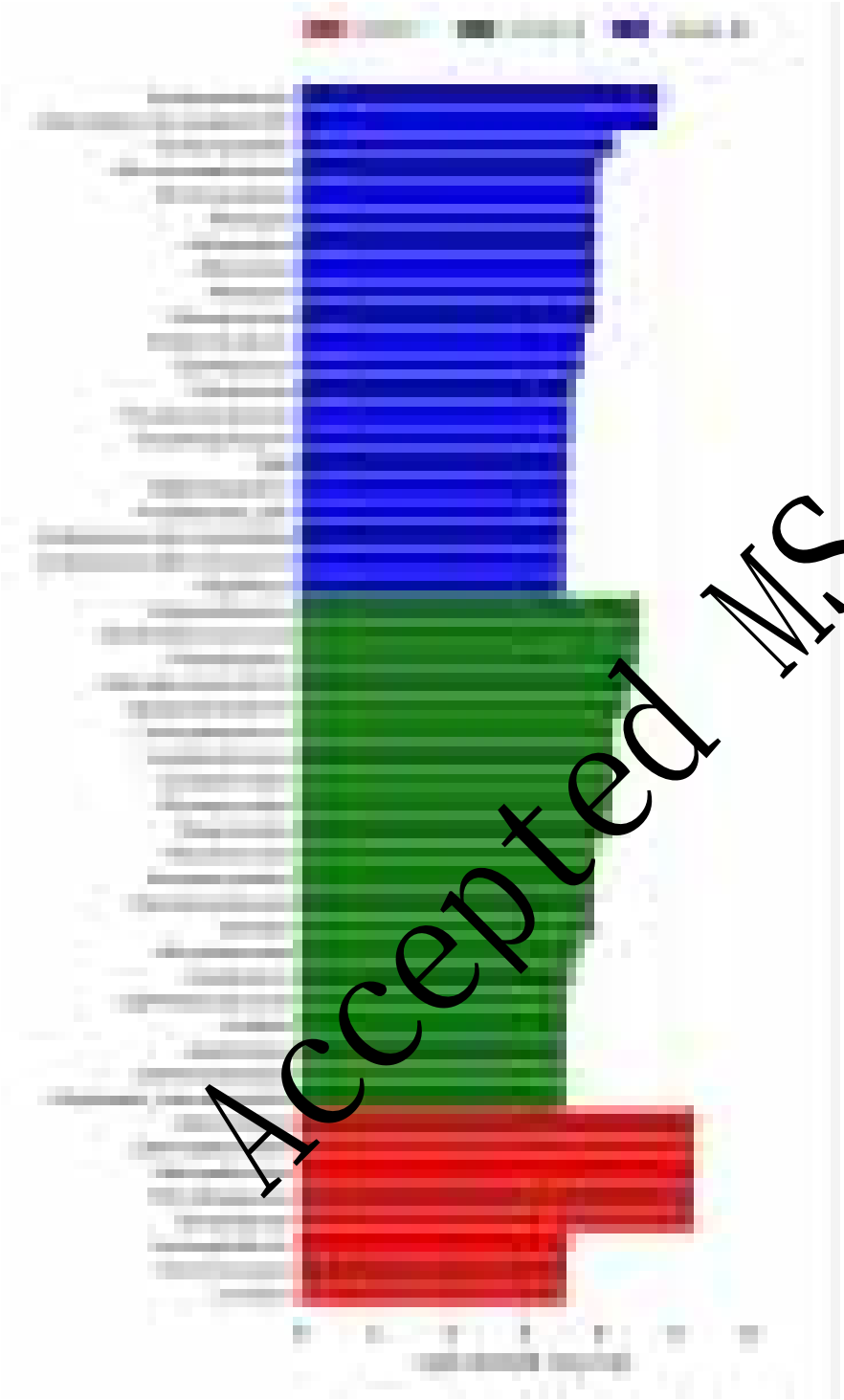

Fig. S2. The logarithmic LDA score of significantly different taxa in different clusters. The values of effect size indicate the degree of difference in relative abundance of bacterial taxon among different clusters.

Fig. S3. Relative abundance of three feature bacterial phyla in cluster I (a), cluster II (b), and cluster III (c) identified from LEfSe analysis. The means and medians in each cluster are shown with straight lines and dotted lines, respectively.

Table S1

Experimental design for studying the effects of MWCNTs on microbial community in riverine sediment contaminated with 2,4-DCP.

Treatment	Components		
Treatment 1 (T1)	Sediment	–	–
Treatment 2 (T2)	Sediment	2,4-DCP (30 mg/kg)	–
Treatment 3 (T3)	Sediment	–	MWCNTs (0.05 mg/g)
Treatment 4 (T4)	Sediment	2,4-DCP (30 mg/kg)	MWCNTs (0.05 mg/g)
Treatment 5 (T5)	Sediment	–	MWCNTs (0.50 mg/g)
Treatment 6 (T6)	Sediment	2,4-DCP (30 mg/kg)	MWCNTs (0.50 mg/g)
Treatment 7 (T7)	Sediment	–	MWCNTs (5.00 mg/g)
Treatment 8 (T8)	Sediment	2,4-DCP (30 mg/kg)	MWCNTs (5.00 mg/g)
Treatment 9 (T9)	Sediment	–	MWCNTs (50.0 mg/g)
Treatment 10 (T10)	Sediment	2,4-DCP (30 mg/kg)	MWCNTs (50.0 mg/g)

Table S2

Estimation of biota-sediment accumulation factor (BSAF) for 2,4-DCP in sediments with different concentration of MWCNTs.

Concentration of MWCNTs in sediment (mg/g)	K_d (L/kg) [a]	BSAF [b]
0	2.6	31.8
5	5.4	15.3
10	8.3	10.0
15	12.6	6.6

[a] Equilibrium partition coefficient (K_d) was calculated from the adsorption isotherms.

[b] BSAF was calculated using log K_{ow} = 3.08, log BCF = 0.85 log K_{ow} − 0.70, and BSAF = BCF/K_d (Jiao et al. 2014, Meng et al. 2018). K_{ow}: octanol-water partitioning coefficient, BCF: bioconcentration factor.

Table S3

P-values from two-way ANOVA for the effects of MWCNTs and 2,4-DCP on the microbial biomass carbon, urease activity, and dehydrogenase activity.

	Microbial biomass C	Urease activity	Dehydrogenase activity
MWCNTs	0.001	0.248	0.020
2,4-DCP	< 0.001	< 0.001	0.053
MWCNTs × 2,4-DCP	0.001	0.148	0.933

Table S4

Alpha diversity index for different treatments.

Treatment	Chao1 index [a]	Shannon index [b]
T1	3620.56	4.36
T2	4447.10	5.47
T3	3824.19	4.00
T4	4736.10	5.30
T5	4384.86	4.50
T6	4114.36	5.27
T7	3740.88	4.45
T8	4082.39	5.30
T9	3534.78	5.00
T10	3421.11	5.11

[a] Chao1 index indicates the number of OTU species.

[b] Shannon index reflects the species diversity that takes into account both the species richness and evenness (Niu et al. 2020).

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