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Carboxyl-modified single-walled carbon nanotubes negatively affect bacterial growth and denitrification activity

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Single-walled carbon nanotubes (SWNTs) have been used in a wide range of fields, and the surface modification via carboxyl functionalization can further improve their physicochemical properties. However, whether carboxyl-modified SWNT poses potential risks to microbial denitrification after its release into the environment remains unknown. Here we present the possible effects of carboxyl-modified SWNT on the growth and denitrification activity of *Paracoccus denitrificans* (a model denitrifying bacterium). It was found that carboxyl-modified SWNT were present both outside and inside the bacteria, and thus induced bacterial growth inhibition at the concentrations of 10 and 50 mg/L. After 24 h of exposure, the final nitrate concentration in the presence of 50 mg/L carboxyl-modified SWNT was 21-fold higher than that in its absence, indicating that nitrate reduction was substantially suppressed by carboxyl-modified SWNT. The transcriptional profiling revealed that carboxyl-modified SWNT led to the transcriptional activation of the genes encoding ribonucleotide reductase in response to DNA damage and also decreased the gene expressions involved in glucose metabolism and energy production, which was an important reason for bacterial growth inhibition. Moreover, carboxyl-modified SWNT caused the significant down-regulation and lower activity of nitrate reductase, which was consistent with the decreased efficiency of nitrate reduction.

Due to the extraordinary structural, electrical, and mechanical properties, single-walled carbon nanotubes (SWNTs) have been used in a wide variety of fields, including medicinal chemistry, electronics, optics, and materials science[1,2]. Nevertheless, previous studies pointed out that the very low solubility is still a major stumbling block in the applications of SWNTs[2,3]. Recently, chemical functionalization has attracted enormous interest, because it has the ability to improve the solubility of nanomaterials[4]. It was reported in the literature that SWNTs could be chemically derivatized with hydrophilic substituents (such as carboxylic acid groups) for medical and biological applications, and these functional groups were able to provide sites for covalent integration of SWNTs into organic or inorganic polymer structures to produce excellent composite materials[5,6]. However, the chemical modification can change the cell-SWNT interactions[7], which suggests the necessity to consider the potential risks of the surface modified SWNT.

Recent studies have indicated that some emerging pollutants such as engineered nanomaterials cause negative effects on biological denitrification, a unique pathway by which fixed nitrogen such as nitrate is converted into dinitrogen gas (N_2)[8]. For example, the presence of quantum dots was observed to affect the denitrification process of *Pseudomonas stutzeri*[9], and the exposure to oxide nanoparticles inhibited the nitrate reduction of mixed culture in activated sludge[10–12]. However, it is still unclear whether carboxyl-modified SWNT can pose the possible risks to microbial denitrification (especially bacterial growth and nitrate reduction) once it has been intentionally or unintentionally released into the environment.

Denitrifying bacteria usually use nitrate as an electron acceptor and sequentially reduce it to N_2. At the same time, to achieve successful denitrification, these denitrifiers need to use organic matter as an electron donor[8]. It has been reported that the denitrifying enzymes, such as nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR), perform the vital functions in these reduction steps[8]. Therefore, it can be concluded that the denitrification capacity of the denitrifier is closely relevant to the gene expressions and catalytic activities of these key enzymes involved in denitrification[13,14].

Here we present the potential effects of carboxyl-modified SWNT on the bacterial growth and denitrification process. Firstly, we adopted scanning and transmission electron microscopes to show the interactions between

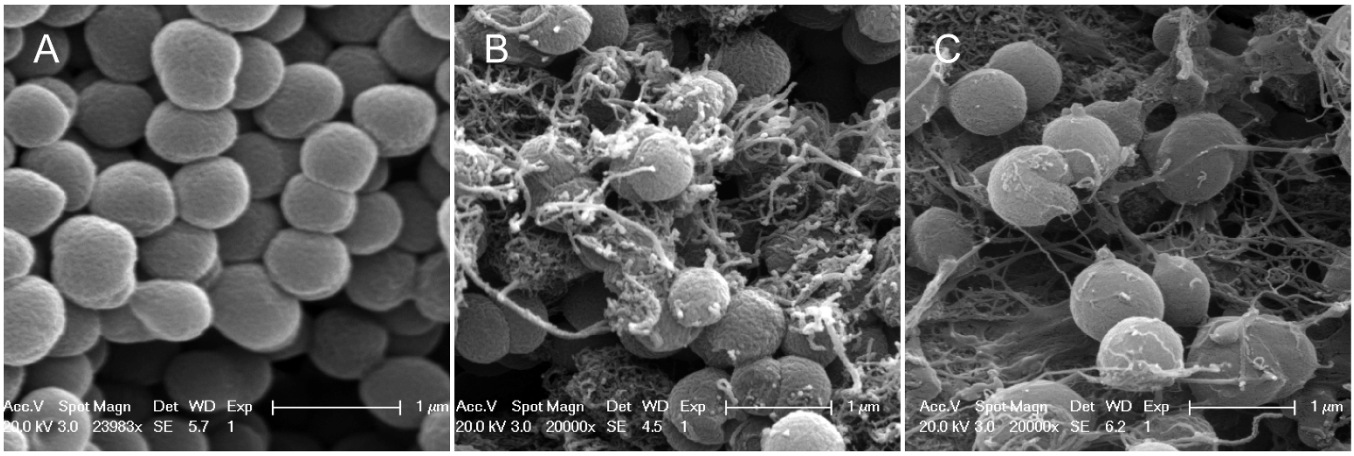

Figure 1 | SEM images of *P. denitrificans* cells in the absence (A) and presence of 50 mg/L non-modified SWNT (B) and carboxyl-modified SWNT (C) after 24 h of exposure.

SWNTs and bacterial cells. Then, lactate dehydrogenase (LDH) release assays were used to indicate the bacterial membrane integrity after exposure to SWNTs. Also, the growth curves and transformations of nitrogen (including nitrate, nitrite, and nitrous oxide) were determined to assess the influences of SWNTs on microbial denitrification. Finally, we conducted the RNA sequencing (RNA-Seq) and reverse transcriptase quantitative PCR (RT-qPCR), and measured the catalytic activities of key denitrifying enzymes in order to reveal the underlying mechanisms of SWNTs affecting bacterial growth and denitrification activity.

Results

Effects of carboxyl-modified SWNT on bacterial cell surface. In this study, scanning electron microscope (SEM) images were used to directly display the cell morphology of *P. denitrificans* exposed to non-modified SWNT and carboxyl-modified SWNT. Figure 1A shows that the bacterial cells of *P. denitrificans* had smooth membrane surfaces in the culture medium without the presence of non-modified SWNT or carboxyl-modified SWNT. When the culture medium contained non-modified SWNT (Figure 1B) and carboxyl-modified SWNT (Figure 1C), these bacterial cells were trapped in the aggregated nanotubes. The membrane integrity measurements further indicated that there was no significant difference in the relative LDH releases between the control and the non-modified SWNT or carboxyl-modified SWNT exposure ($p > 0.05$) (Figure S1, Supporting Information), which was consistent with the SEM observations (Figure 1).

Effects of carboxyl-modified SWNT on bacterial growth. Figure 2 shows the growth curves of *P. denitrificans* in the absence and presence of non-modified SWNT and carboxyl-modified SWNT during 24 h of exposure. We found that there were no significant differences in bacterial density (optical density at 600 nm, OD_{600}) during the adaptation period (0–8 h). Nevertheless, in the exponential growth phase (8–20 h), the growth rates and bacterial densities in the presence of 10 and 50 mg/L carboxyl-modified SWNT became remarkably lower than those in the control. After 24 h of exposure, the average OD_{600} value of *P. denitrificans* in the control was 1.46, which was significantly higher than those exposed to 10 and 50 mg/L carboxyl-modified SWNT (1.35 and 1.24) (Figure 2). It should be noted that the presence of 10 or 50 mg/L of non-modified SWNT had no measurable influences on the bacterial growth during 24 h of exposure.

Effects of carboxyl-modified SWNT on bacterial denitrification. In addition to the surface integrity and bacterial growth, the variations of NO_3^--N, NO_2^--N, and N_2O in the presence of non-modified SWNT and carboxyl-modified SWNT were further investigated. Figure 3 illustrates that compared with the control, the exposure to non-modified SWNT (10 or 50 mg/L) had no negative influences on the transformations of NO_3^--N, NO_2^--N, and N_2O ($p > 0.05$). When *P. denitrificans* was exposed to 10 and 50 mg/L carboxyl-modified SWNT, the nitrate residue concentrations were 24.3 and 44.5 mg/L, respectively, greatly higher than that in the control (2.1 mg/L) (Figure 3A). However, carboxyl-modified SWNT showed no measurable effects on the variations of NO_2^--N, and N_2O ($p > 0.05$), indicating that carboxyl-modified SWNT caused the significant inhibitory effect on the nitrate reduction process of *P. denitrificans*.

Effects of carboxyl-modified SWNT on the transcriptional profiling and denitrifying enzyme activity. Because carboxyl-modified SWNT obviously affect the bacterial growth and denitrification activity, we further explored the transcriptional profiles of *P. denitrificans* in the absence (the control) and presence of 50 mg/L carboxyl-modified SWNT in order to reveal potential mechanisms underlying these negative effects. It can be seen in Table 1 that a total number of 44912380 reads were obtained in this study after raw

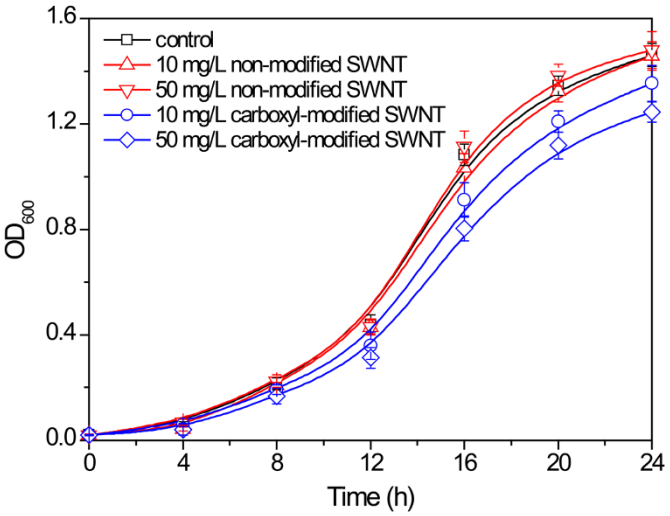

Figure 2 | Growth curves of *P. denitrificans* in the absence (control) and presence of non-modified SWNT and carboxyl-modified SWNT during 24 h of exposure. Error bars represent standard deviations of triplicate measurements.

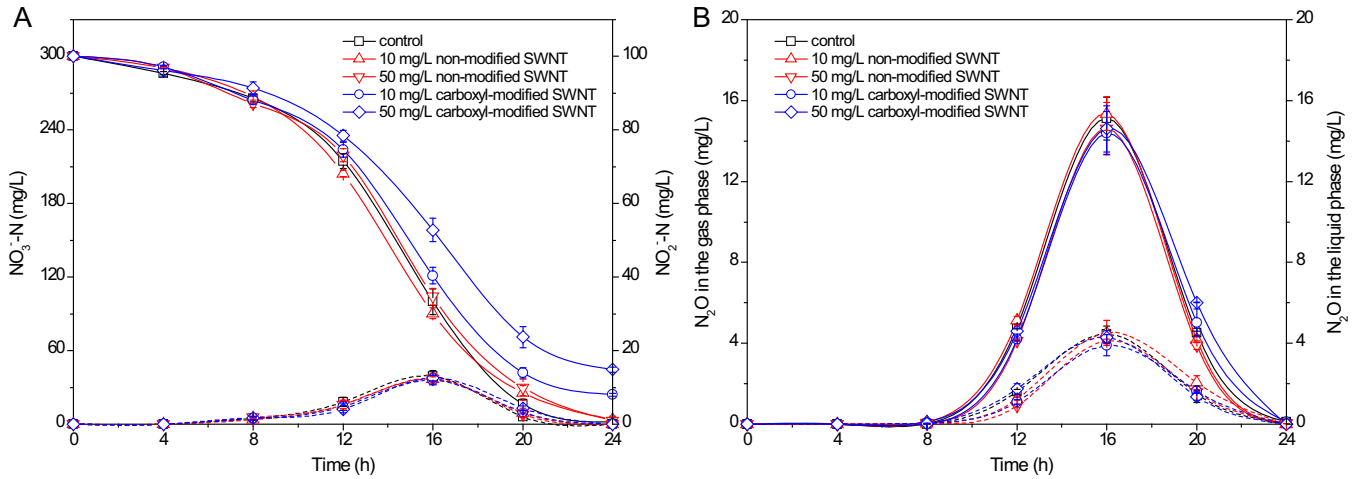

Figure 3 | Transformations of NO_3^--N (A, solid line), NO_2^--N (A, dashed line) (A) and N_2O in the gas (B, solid line) and liquid phases (B, dashed line) in the absence (control) and presence of non-modified SWNT and carboxyl-modified SWNT. Error bars represent standard deviations of triplicate measurements.

sequences processing and filtering. Among these clean reads, 92.8% and 87.7% of the total reads matched to the reference genome in the absence and presence of carboxy-modified SWNT, respectively, suggesting that the sequencing reads could well reflect the transcriptional expressions of *P. denitrificans*.

Gene Ontology (GO) annotation has become a major tool for analysis of genome-scale experiments. Figure 4 illustrates the functional annotation of the clean reads at the GO category of biological process. We observed that the gene expressions were mainly involved in the metabolic processes of organic compound, nitrogen compound, macromolecule, small molecule, and cellular biosynthesis. Meanwhile, there were no significant differences between the control and carboxyl-modified SWNT exposure experiments. Figure 5 further gives the MA plot and volcano plot of genome-wide expression changes. Using the criteria of >2-fold change and FDR < 0.05, 37 significant differentially expressed genes (DEGs) with 26 up-regulations and 11 down-regulations were identified in the presence of 50 mg/L carboxyl-modified SWNT.

The bacterial growth and denitrification of *P. denitrificans* require the uptake and degradation of organic molecules to obtain energy and reducing power[8]. For example, in this study, glucose was used as the sole carbon and energy source. Previous studies indicated that some enzymes, such as phosphoglucomutase and pyruvate kinase, play important roles in the metabolism of glucose[15]. The energy production is observed to be closely related to the key enzymes responsible for the transformation of ATP such as ATPase[16]. Figure 6 shows that the gene expressions of phosphoglucomutase, pyruvate kinase, and ATPase were significantly inhibited by carboxyl-modified SWNT. These results suggested that the presence of carboxyl-modified SWNT caused the negative effects on glucose utilization and energy production processes, which might be an important reason for the growth inhibition.

It is well-know that NAR, NIR, NOR, and N_2OR are the key enzymes responsible for microbial denitrification. Many studies have used *narG*, *nirS*, *norB*, and *nosZ* genes as molecular markers to examine the expressions of NAR, NIR, NOR, and N_2OR, respectively[17]. Thus, we quantified the expressions of *narG*, *nirS*, *norB*, and *nosZ* genes in the absence and presence of 50 mg/L carboxyl-modified SWNT in order to understand their possible influences on microbial denitrification. Figure 7A illustrates that carboxyl-modified SWNT caused the down-regulation of *narG* gene, indicating that the expression of NAR was inhibited by carboxyl-modified SWNT. Moreover, Figure 7B reveals that the presence of carboxyl-modified SWNT significantly decreased the catalytic activity of NAR ($p < 0.05$), which was consistent with the inhibition to nitrate reduction caused by carboxyl-modified SWNT.

Discussion

Engineered nanomaterials usually lead to cell leakage after contact with cell membranes, because their small sizes and high reactivities can easily cause the production of reactive oxygen species (ROS)[18,19]. It is well-known that the ROS formation is mainly resulted from the reduction of oxygen, but microbial denitrification occurs under the anoxic condition. Our results indicated that no matter whether

Table 1 | Summary of the reads mapped to the reference genomes and genes of *P. denitrificans* in the absence (Control) and presence of 50 mg/L carboxyl-modified SWNT

		Control		Carboxyl-modified SWNT	
		Number of reads	Percentage	Number of reads	Percentage
Total number of high-quality reads		23080806	100.0%	21831574	100.0%
Total basepairs of high-quality reads		1881207366	100.0%	1666282416	100.0%
Map to Genome	Total mapped reads	21414916	92.8%	19136498	87.7%
	Unique matches	18935555	82.1%	14784420	67.8%
	Multi-position matches	2479361	10.7%	4352078	19.9%
	Total unmapped reads	1665890	7.2%	2695076	12.3%
Map to Gene	Total mapped reads	15015022	65.1%	13585102	62.2%
	Unique matches	12168362	52.7%	9259014	42.4%
	Multi-position matches	2846660	12.4%	4326088	19.8%
	Total unmapped reads	8065784	34.9%	8246472	37.8%

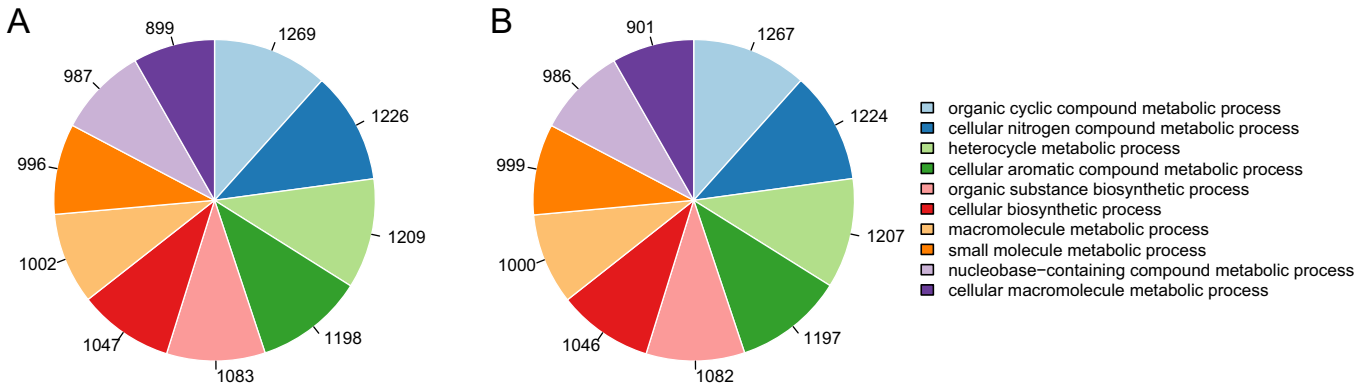

Figure 4 | GO analysis of the sequencing reads from the exposure experiments with (A) and without (B) the presence of 50 mg/L carboxyl-modified SWNT.

SWNT was modified with carboxyl groups or not, its direct contact with *P. denitrificans* did not disrupt cell membranes (Figure 1), and thus had no measurable effects on the cell membrane integrity. Although the cell membrane integrity of *P. denitrificans* was unaffected by the presence of non-modified SWNT and carboxyl-modified SWNT, the increased concentrations of carboxyl-modified SWNT could induce the growth inhibition of *P. denitrificans* (Figure 2). On the other hand, carboxyl-modified SWNT also decreased the rate of nitrate reduction, and thus led to the higher nitrate residue concentration after 24 h of exposure (Figure 3).

It was reported that the dispersion status of nanomaterials plays an important role in their negative effects on living organisms[18,20]. SWNTs can form the aggregation easily in aqueous solution owing to the strong π-π interaction, but the surface functionalization has the ability to enhance the dispersion efficiency of nanomaterials[4]. The zeta potential data showed that the carboxyl modification highly improved the dispersion of SWNTs (Figure S2, Supporting Information), which supported the previous results observed in the literature[4,5]. The highly dispersed SWNTs can attach bacterial surfaces easily, and has the ability to cause the negative influences on bacterial cells[21], which was consistent with the inhibitory effects of carboxyl-modified SWNT on bacterial growth and denitrification activity of *P. denitrificans*.

Previous publications pointed out that bacterial growth and metabolism are closely related to intracellular complex regulations, such as the transcriptional regulation[22,23]. TEM images showed that some carboxyl-modified SWNTs were indeed present inside the bacterial cells (Figure 8). RNA-Seq analysis further revealed that carboxyl-modified SWNT resulted in the significant changes of gene expressions in *P. denitrificans*, indicating that the direct interference with cytoplasmic proteins or with specific promoters affected gene expression. It was reported in the literature that cells could respond to DNA damage by arresting the cell cycle and activating the genes involved in DNA repair[24]. Ribonucleotide reductase is an essential enzyme for DNA synthesis in all prokaryotic and eukaryotic cells, because this key enzyme catalyzes the rate-limiting step in the production of deoxyribonucleoside triphosphates, the precursors for DNA synthesis[25]. It was found that the ribonucleotide reductase genes were subject to transcriptional induction after DNA damage[26]. Our results showed that carboxyl-modified SWNT greatly increased the gene expressions of ribonucleotide diphosphate reductase and ribonucleoside triphosphate reductase (Figure 6), indicating that the presence of carboxyl-modified SWNT led to the DNA damage of *P. denitrificans*.

Biological denitrification is carried out by large numbers of denitrifying bacteria[8]. Among these denitrifiers, only several species of

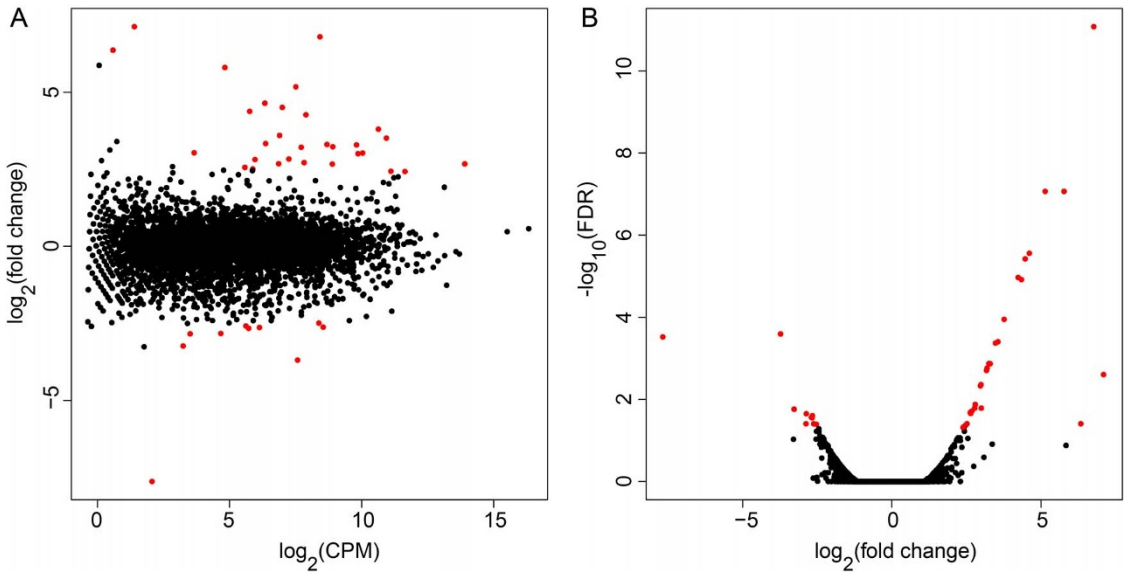

Figure 5 | Distribution of differentially expressed genes (red dots) caused by 50 mg/L carboxyl-modified SWNT in MA plot (A) and volcano plot (B). Red and black dots represent the genes with significantly differential expression (>2-fold change and FDR < 0.05) and with no significant difference, respectively.

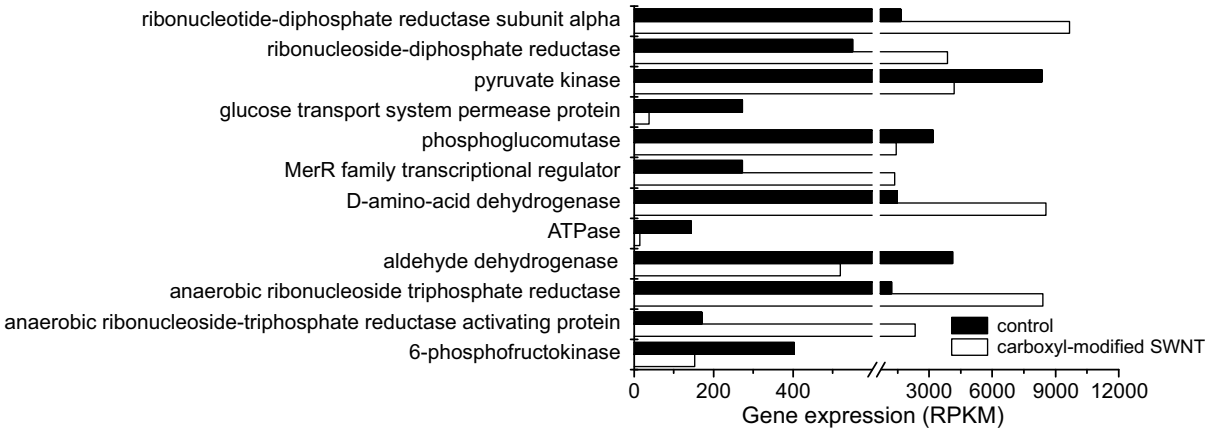

Figure 6 | Gene expressions of key DNA sequences in the absence (control) and presence of 50 mg/L carboxyl-modified SWNT. The gene expression level was calculated using the RPKM (reads per kilobase of exon region per million mapped reads) method.

bacteria such as *P. denitrificans* are able to achieve the complete reduction of nitrate to N_2[27]. It has been reported that *P. denitrificans* can use ATP-binding cassette (ABC) transporters to uptake glucose in the medium, and then consume this sugar to grow and reduce nitrate[8]. However, the gene encoding a glucose transport system permease protein was down-regulated in the presence of 50 mg/L carboxyl-modified SWNT (Figure 6). Concurrently, the gene expressions of the key enzymes involved in glucose metabolism, such as phosphoglucomutase, pyruvate kinase, and ATPase, were also significantly decreased, which was consistent with the observation of glucose utilization being significantly suppressed by 50 mg/L carboxyl-modified SWNT (Figure S3, Supporting Information). These results indicated that the presence of carboxyl-modified SWNT caused the negative effects on glucose utilization and energy production, which was mainly responsible for the growth inhibition. On the other hand, NAR is able to catalyze the reduction of nitrate to nitrite, and we found that carboxyl-modified SWNT inhibited the expression and activity of NAR. Therefore, the decreases in the gene expression and catalytic activity of NAR could lead to the inhibition to nitrate reduction caused by carboxyl-modified SWNT.

In summary, the exposure to carboxyl-modified SWNT had no measurable impacts on bacterial membrane integrity after direct contact with cell membranes. However, the presence of carboxyl-modified SWNT led to the growth inhibition of *P. denitrificans*. TEM images showed that some carboxyl-modified SWNT were present inside the bacterial cells. Transcriptional profiles further indicated that the growth inhibition caused by carboxyl-modified SWNT was due to the bacterial DNA damage and inhibition of gene expressions involved in glucose and ATP metabolism. Also, the presence of carboxyl-modified SWNT resulted in the negative influences on the gene expression and catalytic activity of NAR, which finally caused the decrease in the nitrate reduction of *P. denitrificans*.

Methods

SWNTs and their suspensions. The commercial non-modified SWNT and carboxyl-modified SWNT were purchased from Nanjing Xianfeng Nano Co. (Nanjing, China). The average diameters of these SWNTs were in the ranges of 1–2 nm, whereas their lengths were 0.5–2 μm. To remove residual carbon and metal catalyst, both SWNTs were first heated at 350°C for 3 h and cooled to room temperature. Then, SWNTs were washed using 12 M HCl, sonicated for 1 h, filtered through a 5 μm polytetrafluoroethylene (PTFE) membrane, resuspended in deionized (DI) water repeatedly, and adjusted to neutral pH. The final solution was dried in an oven at 60°C overnight to get SWNT powders[28]. To prepare the stock suspensions, 1 g SWNTs were added into 1 L of Milli-Q water, followed by ultrasonication (25°C, 250 W, 40 KHz) for 1 h to enhance the dispersion.

Cell culture. *Paracoccus denitrificans* (ATCC 19367) was purchased from American Type Culture Collection, and grown in Difco nutrient broth at 30°C and 200 rpm to an optical density at 600 nm (OD_{600}) of 0.8–1.0. Then, the culture of *P. denitrificans* was used as the inoculum.

Effects of SWNTs on bacterial growth and denitrification. To investigate the potential effects of SWNTs, *P. denitrificans* was exposed to 0, 10, and 50 mg/L non-modified SWNT and carboxyl-modified SWNT in a mineral medium modified from

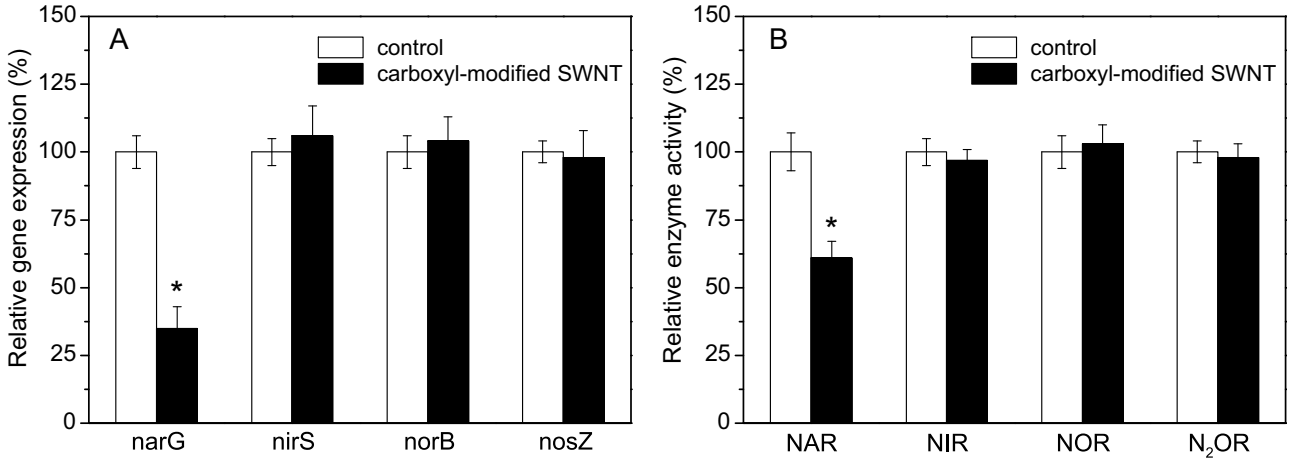

Figure 7 | Gene expressions (A) and relative activities (B) of nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR) in the absence (control) and presence of 50 mg/L carboxyl-modified SWNT. Error bars represent standard deviations of triplicate measurements. Asterisks indicate statistical differences ($p < 0.05$) from the control test.

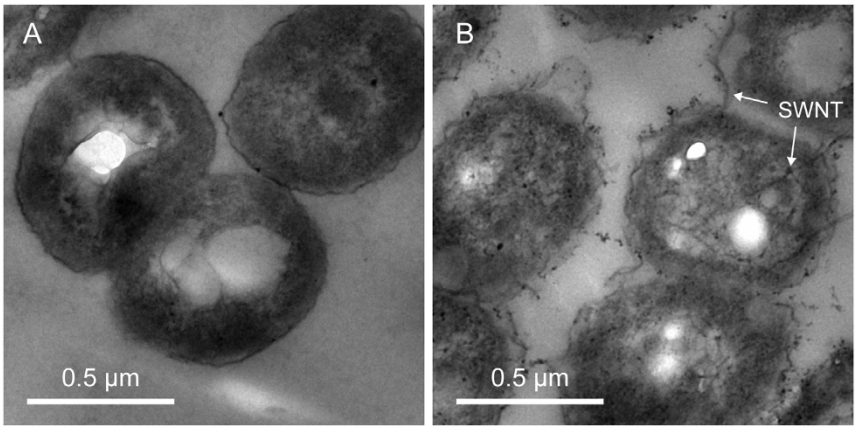

Figure 8 | TEM images of *P. denitrificans* cells in the absence (A) and presence of 50 mg/L carboxyl-modified SWNT (B) after 24 h of exposure.

the literature[27]. The mineral medium contained (per liter) 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.5 g $MgSO_4$, 1.0 g $(NH_4)_2SO_4$, 2.16 g KNO_3, 5.0 g glucose, and 50 μL trace element feed. In particular, the addition of ammonium sulfate was used to preclude assimilatory nitrate reduction, which ensured that nitrate consumption was due to respiration[9]. The trace element feed contained (per liter) 7.4 g $CaCl_2 \cdot 2H_2O$, 1.0 g $MnSO_4 \cdot H_2O$, 3.6 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $CoCl_2 \cdot 6H_2O$, 0.96 g $FeCl_3 \cdot 6H_2O$, 0.027 g $CuCl_2 \cdot 2H_2O$, 0.3 g H_3BO_3, 0.01 g $NiCl_2 \cdot 6H_2O$, and 3.7 g EDTA[10]. The cultures of *P. denitrificans* were inoculated in the medium at an initial OD_{600} of 0.05. Then, these serum bottles were bubbled with argon gas, sealed with rubber stoppers, and placed into a shaker at 30°C and 200 rpm. During 24 h of exposure, the cultures were withdrawn from the serum bottles at intervals of 4 h, and their OD_{600} values were measured via a spectrophotometer (Shimadzu UV-1800, Japan). It should be noted that the OD_{600} values of SWNTs were also determined and subtracted from those of the cultures in order to obtain the OD_{600} values of bacterial cells according to the literature[29]. Meanwhile, the transformations of NO_3^--N, NO_2^--N, and N_2O were examined in order to show the possible effects of SWNTs on microbial denitrification processes.

RNA-Seq analysis. Bacterial cells were harvested in the exponential growth phase (16 h) by centrifugation at 10000 *g* for 10 min at 4°C, and then lysed in TRIzol reagent (Invitrogen) for extraction of total RNA. To avoid DNA contamination, the extracted RNA was treated with DNase I (Ambion) according to the manufacturer's protocol. Thereafter, mRNA was isolated from the DNA-free total RNA using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion), and prepared for Illumina sequencing using the mRNA-Seq Sample Preparation Kit (Illumina) according to manufacturer's instructions. The RNA-Seq libraries were finally sequenced using an Illumina HiSeq 2000. All sequences have been deposited in NCBI SRA database under accession numbers SRX400081 and SRX400082. Raw reads were filtered by removing the reads with (1) sequence adapters, (2) more than 5% 'N' bases, and (3) more than 50% QA ≤ 15 bases. Then, the clean reads were aligned to the reference genome using SOAP2[30], and no more than 3 mismatches were allowed in the alignment for each read. The gene expression level was calculated using the RPKM (reads per kilobase of exon region per million mapped reads) method[31], and the differentially expressed genes (DEGs) were identified based on the criteria of absolute fold change > 2 and FDR < 0.05. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using Blast2GO using default annotation parameters[32].

Quantification of gene expressions of denitrifying enzymes. The gene expressions of key denitrifying enzymes, such as NAR, NIR, NOR, and N_2OR, were determined by the quantification of *narG*, *nirS*, *norB*, and *nosZ* genes via reverse transcriptase quantitative PCR (RT-qPCR). The extracted total RNA described in the "RNA-Seq analysis" section was used to synthesize cDNA at 42°C according to the literature[9]. Thereafter, cDNA was purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction. The qPCR was performed via a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) in a total volume of 20 μL containing 1 × SYBR Green PCR Master Mix, 0.5 μM each primer, and 1 μL of cDNA. The primers and amplification conditions were documented in Table S1 (Supporting Information). All qPCR assays were performed using three replicates per sample, and contained the control reactions without cDNA.

Enzyme activity assays. After 24 h of exposure, bacterial cells were harvested by centrifugation at 10000 *g* for 10 min, washed thrice with 0.1 M phosphate-buffered saline (PBS buffer) (pH 7.4), and resuspended in the same buffer. Then, the resuspended pellets were disrupted by ultrasonication at 4°C for 5 min, and then the cell debris was removed by centrifugation at 12000 *g* for 10 min at 4°C. The crude cell extracts were immediately used for determination of the activities of NAR, NIR, NOR, and N_2OR. The protein concentrations of cell extracts were measured using Protein Assay Kit (Bio-Rad) with bovine serum albumin (BSA) as a standard. The reaction mixture (2 mL) contained 10 mM potassium phosphate buffer (pH 7.1), 10 mM methyl viologen, 5 mM $Na_2S_2O_4$, 1 mM electron acceptor (NO_3^-, NO_2^-, NO, or N_2O), and 100 μL of cell extract[33]. The reaction mixture was incubated at 30°C, and the consumption of electron acceptor were measured every 10 min according to our previous publication[34].

Scanning electron microscopy. SEM images were used to show the surface morphology of bacterial cells exposed to non-modified SWNT and carboxyl-modified SWNT[10]. Briefly, bacterial cells were centrifuged at 1000 *g* for 5 min after 24 h of exposure. The pellets were washed thrice with 0.1 M PBS buffer (pH 7.4), and fixed in 0.1 M PBS buffer (pH 7.4) containing 2.5% glutaraldehyde at 4°C for 4 h. After rinsing twice with 0.1 M PBS buffer (pH 7.4), the pellets were dehydrated in ethanol serials (50%, 70%, 80%, 90%, and 100%, 15 min per step), and then dried in air. Finally, the images were obtained using the FEI Quanta 200 SEM at 20 kV.

Transmission electron microscopy. After exposure to carboxyl-modified SWNT, cells were harvested by centrifugation, washed thrice with 0.1 M PBS (pH 7.4), and fixed in the perfluorocarbon containing osmium tetroxide (1%) for 1 h. After three rinses in pure perfluorocarbon, the samples were dehydrated, embedded, sectioned, stained, and imaged with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

Analytical methods. The zeta potentials of SWNTs were measured via a Zetasizer Nano ZS (Malvern Instruments, UK). The LDH release was examined using the Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer's instruction. The measurements of NO_3^--N and NO_2^--N were conducted according to the Standard Methods[35]. The detailed procedures for determining N_2O in the gas and liquid phases were documented in the literature[36,37]. Briefly, the gaseous N_2O was analyzed using a gas chromatograph (GC, Agilent 7820A) equipped with an electron capture detector (ECD). The dissolved N_2O was measured by GC using a headspace method[37], and the equilibrium temperature and time were 25°C and 3 h, respectively. This GC contained a pre-column (Porapak Q 80/100 mesh, 1 m × 2 mm) and a main column (Porapak Q 80/100 mesh, 3 m × 2 mm). The temperatures of the columns and ECD were 100 and 300°C, respectively, and a mixture of 91% Ar + 9% CH_4 was used as the carrier gas.

Statistical analysis. All tests were performed in triplicate and the results were expressed as mean ± standard deviation. An analysis of variance (ANOVA) was used to test the significance of results and $p < 0.05$ was considered to be statistically significant.

1. De Volder, M. F. L., Tawfick, S. H., Baughman, R. H. & Hart, A. J. Carbon nanotubes: Present and future commercial applications. *Science* **339**, 535–539 (2013).
2. Tasis, D., Tagmatarchis, N., Bianco, A. & Prato, M. Chemistry of carbon nanotubes. *Chem. Rev.* **106**, 1105–1136 (2006).
3. Chen, J. *et al.* Solution properties of single-walled carbon nanotubes. *Science* **282**, 95–98 (1998).
4. Hirsch, A. Functionalization of single-walled carbon nanotubes. *Angew. Chem. Int. Edit.* **41**, 1853–1859 (2002).
5. Peng, H., Alemany, L. B., Margrave, J. L. & Khabashesku, V. N. Sidewall carboxylic acid functionalization of single-walled carbon nanotubes. *J. Am. Chem. Soc.* **125**, 15174–15182 (2003).
6. Du, J. *et al.* The interaction of serum proteins with carbon nanotubes depend on the physicochemical properties of nanotubes. *J. Nanosci. Nanotechnol.* **11**, 10102–10110 (2011).
7. Nimmagadda, A., Thurston, K., Nollert, M. U. & McFetridge, P. S. F. Chemical modification of SWNT alters in vitro cell-SWNT interactions. *J. Biomed. Mat. Res. Part A* **76**, 614–625 (2006).

8. Zumft, W. G. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **61**, 533–616 (1997).
9. Yang, Y., Zhu, H., Colvin, V. L. & Alvarez, P. J. Cellular and transcriptional response of *Pseudomonas stutzeri* to quantum dots under aerobic and denitrifying conditions. *Environ. Sci. Technol.* **45**, 4988–4994 (2011).
10. Zheng, X., Wu, R. & Chen, Y. Effects of ZnO nanoparticles on wastewater biological nitrogen and phosphorus removal. *Environ. Sci. Technol.* **45**, 2826–2832 (2011).
11. Zheng, X., Su, Y. & Chen, Y. Acute and chronic responses of activated sludge viability and performance to silica nanoparticles. *Environ. Sci. Technol.* **46**, 7182–7188 (2012).
12. Chen, Y., Su, Y., Zheng, X., Chen, H. & Yang, H. Alumina nanoparticles-induced effects on wastewater nitrogen and phosphorus removal after short-term and long-term exposure. *Water Res.* **46**, 4379–4386 (2012).
13. Desvergne, B., Michalik, L. & Wahli, W. Transcriptional regulation of metabolism. *Physiol. Rev.* **86**, 465–514 (2006).
14. Siciliano, S. D., Roy, R. & Greer, C. W. Reduction in denitrification activity in field soils exposed to long term contamination by 2,4,6-trinitrotoluene (TNT). *FEMS Microbiol. Ecol.* **32**, 61–68 (2000).
15. Saltiel, A. R. & Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799–806 (2001).
16. Yoshida, M., Muneyuki, E. & Hisabori, T. ATP synthase - A marvellous rotary engine of the cell. *Nat. Rev. Mol. Cell Biol.* **2**, 669–677 (2001).
17. Philippot, L. *et al.* Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the nar, nir, nor and nos genes. *Biochim. Biophys. Acta* **1517**, 436–440 (2001).
18. Colvin, V. L. The potential environmental impact of engineered nanomaterials. *Nat. Biotechnol.* **21**, 1166–1170 (2003).
19. Magrez, A. *et al.* Cellular toxicity of carbon-based nanomaterials. *Nano Lett.* **6**, 1121–1125 (2006).
20. Nel, A., Xia, T., Madler, L. & Li, N. Toxic potential of materials at the nanolevel. *Science* **311**, 622–627 (2006).
21. Bottini, M. *et al.* Multi-walled carbon nanotubes induce T lymphocyte apoptosis. *Toxicol. Lett.* **160**, 121–126 (2006).
22. Stelling, J., Klamt, S., Bettenbrock, K., Schuster, S. & Gilles, E. D. Metabolic network structure determines key aspects of functionality and regulation. *Nature* **420**, 190–193 (2002).
23. Covert, M. W., Knight, E. M., Reed, J. L., Herrgard, M. J. & Palsson, B. O. Integrating high-throughput and computational data elucidates bacterial networks. *Nature* **429**, 92–96 (2004).
24. Khanna, K. K. & Jackson, S. P. DNA double-strand breaks: Signaling, repair and the cancer connection. *Nat. Genet.* **27**, 247–254 (2001).
25. Gon, S. *et al.* A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in *Escherichia coli*. *EMBO J.* **25**, 1137–1147 (2006).
26. Jordan, A. & Reichard, P. Ribonucleotide reductases. *Annu. Rev. Biochem.* **67**, 71–98 (1998).
27. Blaszczyk, M. Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*. *Appl. Environ. Microbiol.* **59**, 3951–3953 (1993).
28. Ghafari, P. *et al.* Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa. *Nat. Nanotechnol.* **3**, 347–351 (2008).
29. Yang, C., Mamouni, J., Tang, Y. & Yang, L. Antimicrobial activity of single-walled carbon nanotubes: Length effect. *Langmuir* **26**, 16013–16019 (2010).
30. Li, R. *et al.* SOAP2: An improved ultrafast tool for short read alignment. *Bioinformatics* **25**, 1966–1967 (2009).
31. Mortazavi, A., Williams, B. A., Mccue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* **5**, 621–628 (2008).
32. Conesa, A. *et al.* Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674–3676 (2005).
33. Kristjansson, J. K. & Hollocher, T. C. First practical assay for soluble nitrous oxide reductase of denitrifying bacteria and a partial kinetic characterization. *J. Biol. Chem.* **255**, 704–707 (1980).
34. Zhu, X. & Chen, Y. Reduction of N_2O and NO generation in anaerobic-aerobic (low dissolved oxygen) biological wastewater treatment process by using sludge alkaline fermentation liquid. *Environ. Sci. Technol.* **45**, 2137–2143 (2011).
35. APHA. *Standard Methods for the Examination of Water and Wastewater,* 20th ed.(American Public Health Association: Washington, DC, 1998).
36. Rosamond, M. S., Thuss, S. J. & Schiff, S. L. Dependence of riverine nitrous oxide emissions on dissolved oxygen levels. *Nat. Geosci.* **5**, 715–718 (2012).
37. Wang, Y. *et al.* Effect of anaerobic reaction time on denitrifying phosphorus removal and N_2O production. *Bioresour. Technol.* **102**, 5674–5684 (2011).

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Author contributions

X.Z. and Y.C. conceived and designed the experiments. X.Z., Y.S., R.W. and M.L. carried out the experiments. X.Z., Y.S., Y.W. and H.H. collected data and performed the statistical analysis. X.Z., Y.S. and Y.C. wrote the paper. All authors discussed the results and reviewed the manuscript.

Additional information

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