Supporting Information

The co-effect of ampicillin and multi-walled carbon nanotubes on activated sludge in sequencing batch reactors: the microbial status, microbial community structure and ARGs propagation

Wenlin Zhou[1], Yan Wang [1*], Min Wang[1], Binghong Qian[1], Li Li[1], Baoyu Gao[1]

1. *Shandong Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, Jinan 250100, P.R. China*

Figure and Table Captions

[*] Corresponding author. Tel.: +86 531 88361812; fax: +86 531 88364513
E-mail address: wangyan_sdjn@aliyun.com (Yan Wang)

Supplemental S1: The size distribution and morphology of MWCNTs

In order to investigate the aggregation of MWCNTs in water, the samples of the MWCNTs stocking suspension (0.1mg/L) was taken at ten minutes and 240h after the ultrasound treatment, carried out to disperse the MWCNTs in water to measure the distribution and morphology.

Dynamic Light Scattering (DLS) (Malvern Zetasizer Nano ZS90 equipment (Malvern Instruments, UK) was used to measure the size distribution of MWCNTs in the stocking suspension. The apparatus equipped with a He-Ne laser with a wavelength of 633 nm. The scattering angle used for the measurements was 90°. The samples were directly put in the tested cuvettes without any further treatment. All samples were repeated more than three times.

Transmission electron microscopy (TEM, Tecnai G2 F20, USA) was used to measure the morphology of MWCNTs in the stocking suspension. Drop the sample onto a copper wire directly and test at the accelerating voltage of 200kV.

Supplemental S2: The adsorption experiment of MWCNTs

The 0.2g MWCNTs were weighed and dissolved in 180mL distilled water, the solution was dispersed by 30-min of ultra-sonication (20 ℃, 250 W, 40 kHz) and 2-h Ultrasonic cell crusher (SCIENTZ，China). The 2mg AMP were weighed and dissolved in 20mL distilled water, after fully dissolving. The MWCNTs and AMP solution were mixed together and vibrated on a shaker (SHA-B Digital Display Thermostatic Water Bath Oscillator，China) for 24 h. The samples were taken to measure the concentration of AMP at different interval minute. The concentration of AMP was analyzed by HPLC (Thermo Fisher Scientific, USA). The measurement conditions are as follows: mobile phase and ratio (Acetonitrile:H_2O=20:80), column temperature (35℃), retention time (3.9-4.0 minutes), flow velocity (1ml/min) and the wavelength (220nm) . Before test, all samples were filtered through a 0.22 m filter.

Supplemental S3: Preparation of the synthetic wastewater

Considering the COD value, total nitrogen and phosphorus concentration in the municipal wastewater is in the range of 250 to 800 mg/L, 20 to 70 mg/L and 4 to 12 mg/L according to the references[1, 2], the synthetic municipal wastewater was prepared by dissolving CH_3COONa, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$, and $FeCl_3 \cdot 6H_2O$ etc. to DI water according to the reference[3-5]. The details of the content of each material were showed in Table S1.

Supplemental S4: Celluar viability and proliferation assay

AlamarBlue (AB) is a redox indicator that can generate absorbance changes and fluorescence signals based on microbial metabolic activity. During microbial proliferation, the ratio of NADPH/NAD, FADH/FAD, FMNH/FMNP and NADH/NAD increases in bodies, which are in a reductive environment, dye ingested in bacteria is reduced by these metabolic intermediates and released in vitro and dissolved in solution, changing the solution from a non-fluorescent blue to a fluorescent pink. Celluar proliferation was measured with AlamarBlue bacterial activity detection kit (AB, China). Briefly, Cells were seeded onto 96-well polystyrene black plates (TPP® tissue culture plates, Trasadingen, Switzerland) at a concentration of 1×10^4 cells/well(bacterial solution: dye=50:1).The cells were incubated at 37 °C for 4 h in Oscillating incubator, followed immediately by measuring the fluorescence intensity (EX/EM:550/590nm) using Spark multifunctional microplate reader (Tecan，Austria).

Supplemental S5: Live/Dead cells assessment

The live/Dead cells were determined using the living cell dye Calcein-AM (EX/EM: 490/515nm, green fluorescence, captured in 2 channels) and the dead cells dye Propidium Iodium (EX/EM: 535/617nm, red fluorescence, captured in 4 channels). The live/dead staining procedure was performed according to the manufacturer's instructions. Before staining, the cells at a concentration of 1×10^7 cells/well were washed with PBS buffer, then were dispersed using a vortexer. Each sample was stained using the pre-mixed solution (Calcein-AM dye/propidium iodide/Hank's balanced salt solution=2μL: 1μL: 0.5mL) available in kits. After incubation, the samples were rinsed in PBS and passed through a 200 mesh sieve then observed using ImageStream× Mark II flow cytometry (Amnis, USA) with 488nm exciter.

Supplemental S6: Cell membrane permeabilization

The damage and integrity of the cell membrane was determined using the PI stain, which can only penetrate permeabilized membranes due to its large size and negative charge. Cells with permeability emit red fluorescence by 488nm laser excitation after the uptake of PI dye, which were captured in 4 channels. The fluorescent intensity of CH04 indicated strongly the loss of membrane integrity. In the experiments, the stain was diluted with PBS at the ratio of 1:100, and samples were immersed for 20 min and then rinsed in PBS. Disperse the cells using a vortexer before FCM analysis.

Supplemental S7: Cell death modes

Detecting the externalization of phosphatidylserine (PPS) in apoptotic bacteria using recombinant Annexin V conjugated to green-fluorescent FITC dye (captured in 2 channels) and dead bacteria using propidium iodide (captured in 4 channels) with a red-fluorescence. Annexin V is a Ca^{2+} dependent phospholipid binding protein with a molecular weight of 35.8 KD. It can bind to PPS with high affinity in the process of apoptosis. Annexin V-FITC staining can identify apoptosis at an early stage as well as the late stages of cell death resulting from either apoptotic or necrotic processes. Staining procedure: briefly, using PBS buffer to prepare single cells suspension 100μL with a concentration 1×10^6-1×10^7 cells/mL, and then incubating for 15 minutes after adding the 1μL Annexin V-FITC at 37℃ in the dark, followed by adding 2μL PI incubating for 5 min at 37℃ in the dark and washed with the PBS immediately and analysis, disperse the cells using a vortexer before analysis.

Supplemental S8：DNA extraction

Sludge samples harvested at the end of the exposure experiments (240 h) were immediately stored at -20℃ until their DNA was extracted. Microbial genomic DNA was extracted from 0.5g frozen sludge samples at Personal Biotechnology Co., Ltd (Shanghai, China) using the Mag-Binding ® Soil DNA Kit (Omega Bio-tek, Norcross,GA, USA) according to the protocol provided by the manufacturer. The integrity of extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA,USA) and checked by agarose gel electrophoresis.

Supplemental S9: Microbial Community Structure and ARGs Analysis

The genomic DNA was accurately quantified for polymer-ase chain reaction (PCR) with the Qubit™ssDNA Assay Kit in a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The hy-pervariable V3-V4 region of bacterial 16S-rRNA gene high-throughput sequencing was amplified by nested PCR with the primer 338F (5′- ACTCCTACGGGAGGCAGCA -3′) and 806R (5′- GGACTACHVGGGTWTCTAAT -3′). The reaction mixture (25 μL) contained 5 μL of 5×reaction buffer, 5 μL of 5 × GC buffer, 2 μL of dNTP (2.5 mM), 1 μL of each primer (10 μM), 2 μL of template DNA, 0.25 μL of Q5 High-Fidelity DNA Polymerase (NEB), and 8.75 μL of ddH2O. The temperature cycle consisted of 98 °C for 1 min, followed by 30 cycles of 98 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×300 bp sequencing was performed using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Personal Biotechnology Co., Ltd (Shanghai, China). ARGs (*bla*$_{FOX}$, *bla*$_{DHA,}$ and *Lak*), twomobile genetic elements (*intI1* and *intI2*) and 16S-rRNAwere quantified by fluorescence quantitative PCR (TIB 8600, China) at Personal Biotechnology Co., Ltd (Shanghai, China). The corresponding primers for PCR are given in Table S3 and the primer amplification efficiency were listed in Table S4. The qPCR reaction mixture comprised 1 μL of DNA template, 0.10 μL of each 20 pM primer (Personalbio,

Shanghai,China), 10 μL of SuperReal PreMix Plus (Tiangen, Beijing, China), and 8.5 μL of RNase-free water. The temperature program for the qPCR conditions was as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and a final step for a melting curve. Product specificity was confirmed by melting curve analysis and visualization in agarose gels. Sterile water was used as the negative control in every run. For the standard curves, the square correlation coefficient (r^2) was > 0.99 and the amplification efficiency ranged between 95% and 110%.

Supplemental S10: Extraction and analysis of EPS

The sludge samples (10 mL) were placed in 50mL centrifuge tubes, and subsequently centrifuged at 8000r for 5 min. The supernatant was poured off and the sediments at the bottom of the centrifuge tubes were re-suspended in a 0.05% (w/w) NaCl solution. The samples were sonicated at 20 kHz for 2 min, subsequently heated at 60℃ for 30 min, the suspension was centrifuged at 10000r for 15min, and the supernatant was collected as EPS. Finally, all the EPS fractions were filtered through 0.45μm membrane filter before measurement and subsequently stored at -20℃. The polysaccharide of EPS was measured by anthracone-sulfate colorimetry using UV-vis at wavelength of 620nm.

Supplemental S11: Intracellular ROS accumulation

The activated sludge was centrifuged at 10000g for 10min and rinsed with a 0.85% NaCl solution (w/w) three times. The sediments at the bottom of tubes were resuspended in a 0.85% NaCl solution and then incubated with DCFH-DA (used after 1:1000 dilution) for 30min, then washed off the excess probe with 0.85% NaCl solution and dispersed the cells using a vortexer. Finally, ROS generation was determined by a fluorescence spectrometer (FL-4600, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Table S1: Composition and concentration of the synthetic wastewater

Reagent	Concentration(mg/L)	Reagent	Concentration(mg/L)
CH_3COONa	769	$CaCl_2$	0.203
$(NH_4)_2SO_4$	284	$MgSO_4 \cdot 7H_2O$	5.07
KH_2PO_4	28	Yeast Extract	30
Trace elements			
$ZnSO_4 \cdot 7H_2O$	0.44	$CoCl_2 \cdot 6H_2O$	0.42
$CuSO_4 \cdot 5H_2O$	0.391	$FeCl_3 \cdot 6H_2O$	2.42
$MnCl_2 \cdot 4H_2O$	0.275	$NaMoO_4 \cdot 2H_2O$	1.26

Table S2: SBR operation parameter table

Operation Temperature	25℃
Operation Cycle	**12h** Including: 0.2h influent period, 2h anoxic period (DO <0.5 mg/L), 7.5h aerobic period (DO>3 mg/L), 1h of settling, 0.5h discharge period(6L) and 0.8h idle period
Sludge Retention Times	15d
Dissolved Oxygen	>3 mg/L
MLSS	3500 ± 100 mg/L

Table S3：Primer pairs used to quantify the 16S-rRNA gene, antibiotic resistance genes and the mobile genetic element *intI1* and *intI2*

Target Genes	Primer	Primer Sequence(5'to3')	TM(℃)	Amplimer(bp)
16S	*16S-338F*	ACTCCTACGGGAGGCAGCA	55	181
	16S-519R	ATTACCGCGGCTGCTGG		
bla_{DHA}	bla_{DHA}*-F*	TTATCTCACACCTTTATTACTG	60	139
	blaDHA-R	TATCTTTTGAGGCGGATT		
bla_{FOX}	*blaFOX-F*	CATTATCCAGCCGATGCTCAAGG	60	186
	blaFOX-R	TAGGCACCGAGGGTCGCAGTCA		
Lak	*Lak2-FP*	GGGAATGCTGGATGCACAA	60	67
	Lak1-RP	CATGACCCAGTTCGCCATATC		
intl1	*intl1-F*	CCTCCCGCACGATGATC	55	284
	intl1-R	TCCACGCATCGTCAGGC		
intl2	*intl2-F*	TTATTGCTGGGATTAGGC	50	233
	intl2-R	ACGGCTACCCTCTGTTATC		

PCR response procedures:

95℃ 5min

95℃ 15sec } 40 cycles

60℃ 30sec

Table S4：Primer amplification efficiency

Gene	Y-Intercept	Slope	R	E
16S	36.36	-3.32	0.9983	100.00%
bla_{DHA}	42.74	-3.9	0.999	80.60%
bla_{FOX}	41.49	-3.88	0.998	81.00%
Lak	39.95	-3.73	0.9965	85.30%
intl1	41.06	-3.83	0.9968	82.30%
intl2	38.56	-3.89	0.9978	80.80%

Table S5: Acronyms and full name of the terms

Full name	References	acronyms
ampicillin	26	AMP
multi-walled carbon nanotubes	2,5	MWCNTs
sequencing batch reactors	5	SBR
antibiotic resistance bacteria	13	ARB
antibiotic resistance genes	13	ARGs
nanoparticles	16	NPs
wastewater treatment plants	22	WWTPs
mixed liquor volatile suspended solid	5	MLVSS
mixed liquor suspended solid	5	MLSS
specific oxygen utilization rate	5	SOUR
total organic carbon	5	TOC
propidium iodide	6,30	PI
calcein-acetoxymethyl ester	11	Calcein-AM
fluorescein-isothiocyanate	67	FITC
polymerase chain reaction	9	PCR
extracellular polymeric substances	38,59,60,67	EPS
reactive oxygen species	5,40,41	ROS
2', 7'-dichlorofluoresceindiacetate	40,41	DCFH-DA
flow cytometer	30,53	FCM

Section S1:

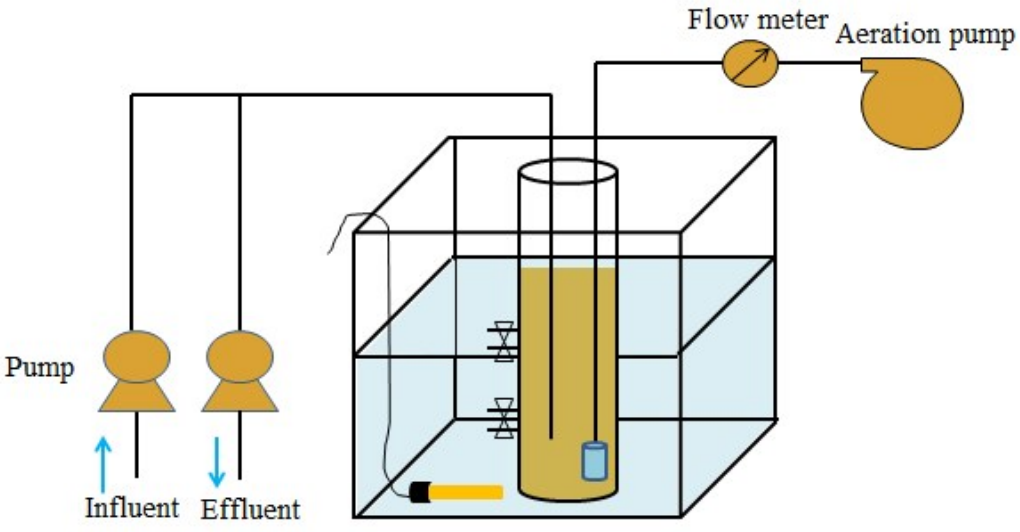

Fig. S1 Experimental device of a laboratory scale SBR

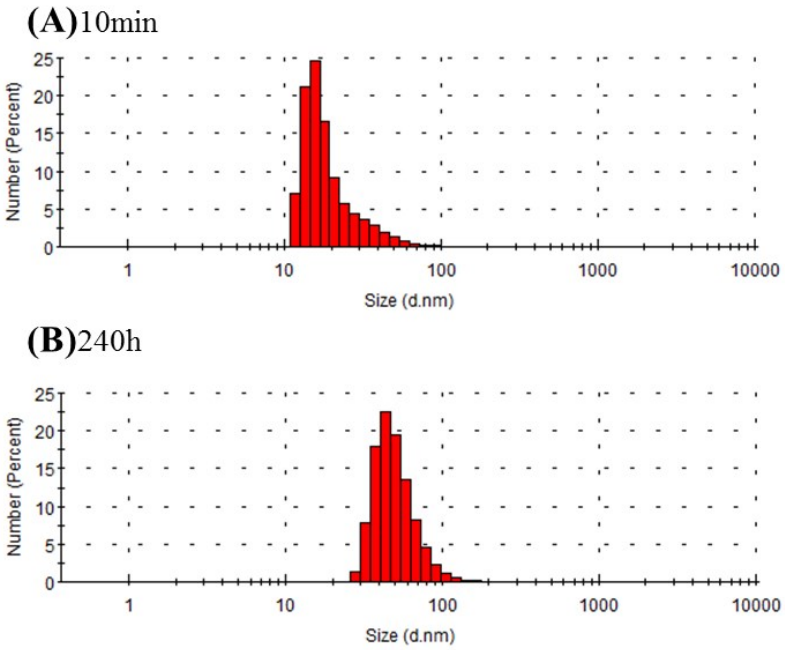

Fig. S2 Particle size distribution of MWCNTs suspensions (100mg/L) in simulated wastewater at (A)10 min and (B)240h after ultrasound under (polydispersity index (PDI) is less than 0.5)

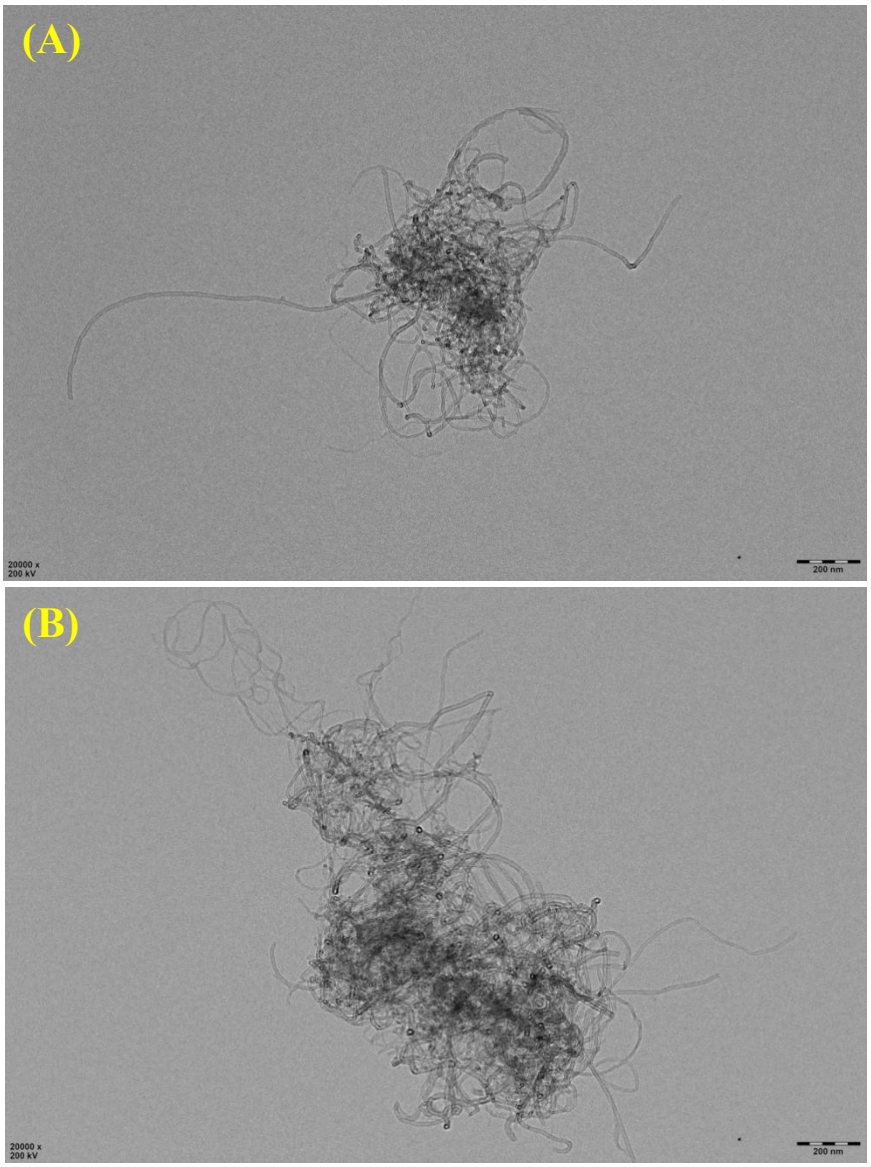

Fig. S3 TEM micrograph of MWCNTs in simulated wastewater

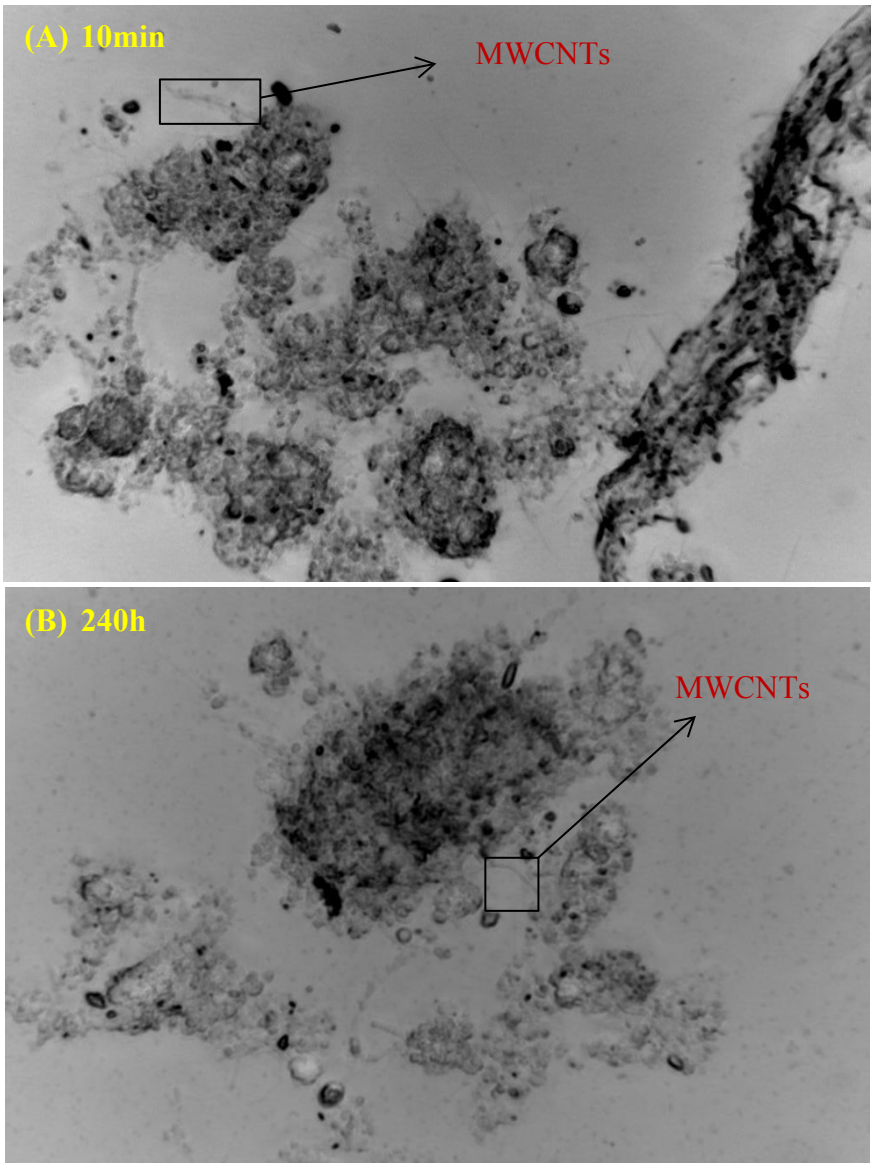

Fig. S4 Optical micrograph of MWCNTs with activated sludge in SBR

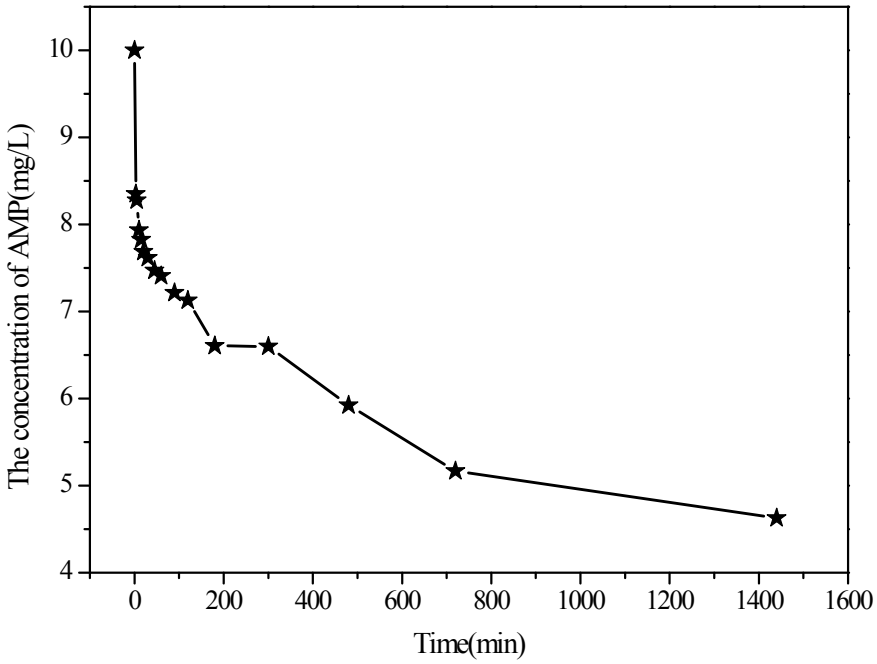

Fig. S5 Adsorption curve: 200mL MWCNTs (1g/L) dispersion liquid adsorbed 10mg/L AMP solution.

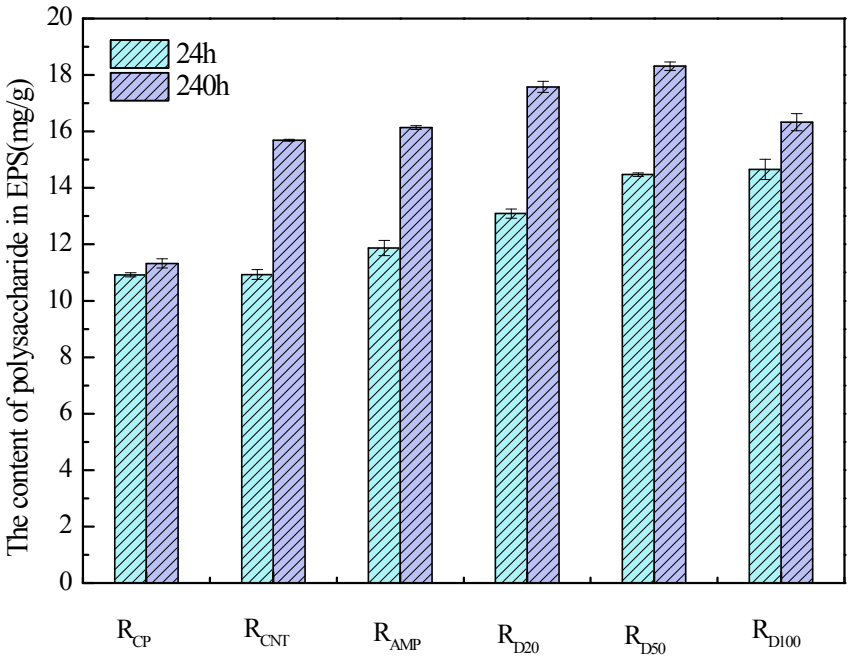

Fig. S6 Content of polysaccharide in EPS after 24h and 240h exposure

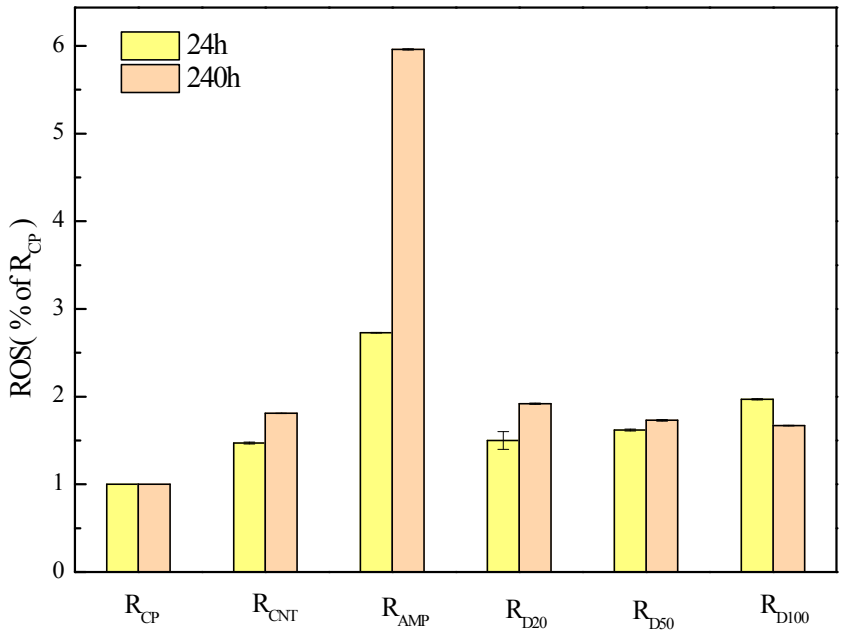

Fig. S7 ROS production after 24h and 240h exposure

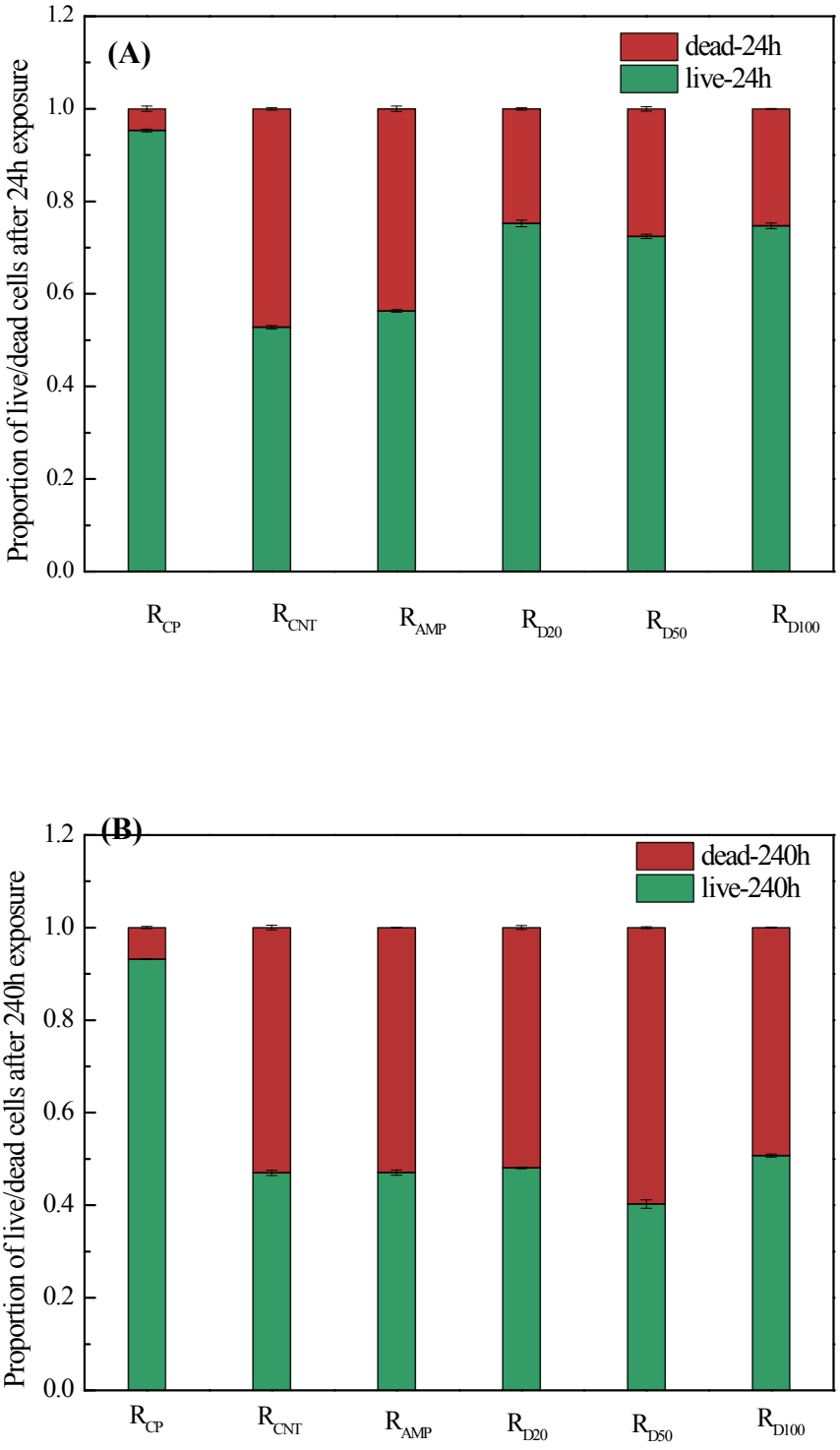

Fig. S8 Proportion of live/dead cells (A) after 24h exposure and (B) after 240h exposure

Reference:

1. X. Yang, Q. He, F. Guo, X. Sun, J. Zhang and Y. Chen, Impacts of carbon-based nanomaterials on nutrient removal in constructed wetlands: Microbial community structure, enzyme activities, and metabolism process, *J Hazard Mater*, 2021, **401**, 123270.
2. M. Chen, X. Zhao, C. Wang, S. Pan, C. Zhang and Y. Wang, Electrochemical oxidation of reverse osmosis concentrates using macroporous Ti-ENTA/SnO2-Sb flow-through anode: Degradation performance, energy efficiency and toxicity assessment, *J Hazard Mater*, 2021, **401**, 123295.
3. M. Gao, F. Gao, B. Ma, N. Yu, Z. She, C. Zhao, L. Guo, Y. Zhao, S. Li and C. Jin, Insights into long-term effects of amino-functionalized multi-walled carbon nanotubes (MWCNTs-NH2) on the performance, enzymatic activity and microbial community of sequencing batch reactor, *Environ Pollut*, 2019, **254**, 113118.
4. M. Jiang, L. Feng, X. Zheng and Y. Chen, Bio-denitrification performance enhanced by graphene-facilitated iron acquisition, *Water Res*, 2020, **180**, 115916.
5. Y. Qu, Q. Ma, J. Deng, W. Shen, X. Zhang, Z. He, J. D. Van Nostrand, J. Zhou and J. Zhou, Responses of microbial communities to single-walled carbon nanotubes in phenol wastewater treatment systems, *Environ Sci Technol*, 2015, **49**, 4627-4635.