Supporting Information to

Nanoplastics Disturb Nitrogen Removal in Constructed Wetlands: Responses of Microbes and Macrophytes

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Summary

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The following is included as additional supplementary materials for this paper:

Text S1. Preparing PS suspension

Raw PS suspension was 5ml and 2.5% w/v (approximatively 25 mg/ml). To prepare the 10 and 1000 μg/L PS suspension, 4.8 μL and 480 μL raw PS suspension were added into 12L synthetic wastewater and blended.

Text S2. Synthetic wastewater

The compositions of synthetic wastewater simulating the influent of WWTPs were shown as following: one hundred liters of synthetic wastewater contained 264 ml concentrated feed, 72.6 ml phosphorus stock solution, 7.64 g ammonium chloride, 0.91g nitrate, 2 ml trace element feed and 10 ml acetic acid. The concentrated feed contained (g/L): 12.89 peptone, 4 yeast extract, 34 $MgCl_2 \cdot 6H_2O$, 20 $MgSO_4 \cdot 7\ H_2O$ and 9 $CaCl_2 \cdot 2\ H_2O$. The phosphorus stock solution contained (g/L): 33 K_2HPO_4 and 29 KH_2PO_4. The trace element feed contained (g/L): 1.50 $FeCl_3 \cdot 6H_2O$, 0.03 $CuSO_4 \cdot 5H_2O$, 0.12 $MnCl_2 \cdot 4H_2O$, 0.06 $Na_2MoO_4 \cdot 2H_2O$, 0.12 $ZnSO_4 \cdot 7H_2O$, 0.15 $CoCl_2 \cdot 6H_2O$, 0.18 KI, 0.15 H_3BO_3 and 10 ethylenediamine tetraacetic acid (Zheng et al. 2011a). The synthetic wastewater concentration is about 200 mg/L COD, 30 mg/L total nitrogen (TN), 20 mg/L NH_4^+-N, and 1.5 mg/L NO_3^--N.

Text S3. The culture and operation of wetland reactors

Prior to starting the formal experiment, all the microcosms were fed with the synthetic wastewater for 4-month pre-incubation in order to culture the biofilm and plant shoots. The water level was attuned to be 5 cm below the gravel bed surface. Each microcosm was worked in batch mode, and the hydraulic retention time (HRT) was 5 days for each batch. Synthetic wastewater was fed into the microcosm via the punched pipe intersecting the reactor from bottom to top, and gravity drained via a tap on the bottom.

Text S4. Preparing samples for CLSM assays

After a 180-day of exposure treatment, 5 g wet gravel sample was respectively sampled from each microcosm and added to 30 ml phosphate buffer solution (PBS) in 100 ml conical flasks to strip the biofilm under shaking at 200 rpm for 1 h. The biofilm was cultured by synthetic wastewater containing PS labelled fluorescent under 25 oC and shaking at 100 rpm in batch model (HRT=5 days). After each batch, 10 μL of biofilm suspension were sampled for cleaning using PBS and centrifugation at 4000 rpm, three times, in the 1.5 ml centrifuge tube. Subsequently, treated biofilm was dispersed by 1 ml PBS in the centrifuge tube. Before observing, 10 μL treated biofilm suspension was placed the glass slide and covered the cover glass. All the microscopic observations and images acquisition were performed by a CLSM (TCS SP8 CSU, Leica, Germany) with ×63 oil-immersion objective using Quick-LUT (Look Up Table) function to set the pixel saturation limits. For the formation of 3d map, we completed it by using the layer scanning function of the software. First, we determined the microorganism needing to be scanned, then determined the range of layer scanning, namely the bottom layer and the top layer, and then set the number of images of layer scanning (we took the 30 layers in this study). After the layer scanning, 3d imaging was performed in software.

Text S5. Measurements of AMO, NAR and NIR activities

AMO activity [1]: The AMO activity was measured by reducing NH_4^+-N to NO_2^--N with minor modification. The 10g gravel sample was added into 50ml conical flask, and mixed with the 20 ml mixture PBS (NaCl: 8.0 g/L; KCl: 0.2 g/L; Na_2HPO_4: 0.2 g/L; NaH_2PO_4: 0.2 g/L; $(NH_4)_2SO_4$: 1 mM; $KClO_3$: 10 mM) (pH=7.4). The conical flask was shaken (150 r/min 4h) in a water bath at 37 °C in the dark, and then added 10ml 2M KCl solution into in conical flask. The mixture solution was also shaken at 200r/min for 5min to lixiviate nitrite. The solution was centrifuged for 5 min at 4000 rpm. Finally, the supernatant was measured spectrophotometrically at 540 nm. The results were

expressed as mg NO_2^--N·g^{-1} gravel·h^{-1}.

NAR and NIR activity [2, 3]: The NAR and NIR activities were measured by NO_2^--N production and NO_2^--N residual after substrate reduction with minor modification. Briefly, the 10g gravel sample was added into 50ml conical flask, and mixed with the 20 ml 0.01M PBS (pH=7.4) to sharply shake for 15 min at 200 r/min and picked out all gravel. The mixture was washed 3 times with 0.01M PBS, and then sonicated at 20 kHz 4°C for 5 min to break down the cell structure. The cell debris was centrifuged at 12000 rpm 4°C for 10 min and the crude extracts in supernatant were obtained for measuring enzymatic activities. The NAR and NIR activities were determined using methyl viologen as electron donor. A volume of 300 μL of crude extracts was added to start the reaction in a cuvette containing 0.01 M phosphate buffer (pH 7.4), 1 mM $NaNO_3$ or $NaNO_2$, 1 mM methyl viologen and 5 mM sodium hyposulfite ($Na_2S_2O_4$) in a final volume of 2 mL. The incubation was conducted at 30 ^oC for 30 min, followed by measuring the produced or remained nitrite in NAR and NIR activities assays. The specific activities of NAR and NIR were expressed as the produced mg N/(g·gravel·h).

Text S6. Measurements of LDH release and ROS production

The cell membrane integrity of microorganisms was measured using a LDH kit (Nanjing Jian-cheng Technolgy Co., Jiangsu, China). 15 mL of PBS (pH=6.8) was added to a conical flask containing 10 g of gravel, which was thoroughly shaken (150 rpm) for 5 min at 25 ^oC, the supernatant was used to determine the LDH release according to the instructions of the LDH kit [4].

ROS production was determined by the dichlorodihydrofluorescein (DCF) assay method according to the procedure reported in the literature [5, 6]. Briefly, the 10g gravel sample with biofilm was added 15ml PBS to sharply shake for 15 min at 200 r/min and picked out all gravel. the mixture was first centrifuged at 10,000g for 10 min and was washed with a 0.85% (w/v) NaCl solution. The collected pellets were then re-suspended in a 0.85% (w/v) NaCl solution and incubated with 20

μmol/L H_2DCF-DA (Molecular Probes, Invitrogen) for 30 min. The mixed liquor was transferred into 96-well microtiter plates for fluorescence spectroscopy at excitation/emission wavelengths of 495/525 nm.

Text S7. Microbial community structure analysis

For the characterization of microbial community, gravel samples (200 g) were composed from three depths (10, 25 and 40 cm) of wetland reactors at the end of long-term exposure experiment, respectively. The gravel samples collected from three depths were mixed for DNA extraction using E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols. The DNA concentration and purity was quantified with TBS-380 and NanoDrop2000, respectively. DNA quality was examined with the 1% agarose gels electrophoresis system. PCR amplification of the 16 S rRNA gene variable V4 region of extracted DNA followed with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with small adjustments according to the literature [7, 8].

Text S8. Analysis of antioxidant enzyme activities, Malonaldehyde (MDA) and root activity

For plant enzyme extraction, 0.2 g of fresh leaf was finely ground into seriflux by the mortar in ice bath. The seriflux was homogenized in 3 mL of 50 mM PBS (pH = 7.0, 1 mM ethylene diamine tetraacetic acid (EDTA) and 2% PVP). Subsequently, the homogenate was placed to a 10 ml centrifuge tube and centrifuged at 10000 rpm for 15 min at 4 ^{o}C, and then the supernatant was immediately analysed for antioxidant enzyme activities.

Superoxide dismutase (SOD) (EC 1.15.1.1) was determined by measuring the ability of SOD enzyme to depress the photochemical reduction of nitro blue tetrazolium (NBT), using a method developed by Giannopolitis and Ries [9]. The reaction solution (3 mL) was illuminated for 15 min under fluorescent lamps (15W), which contained K-phosphate buffer (pH 7.8), 75 nM NBT, 2 nM riboflavin,

13 mM methionine, 0.1 mM EDTA and 100 µL of enzyme extract. One unit of enzyme activity was regarded as the amount of SOD enzyme that inhibited the NBT in the photochemical reduction by 50% at 560 nm.

Catalase (CAT) (EC1.11.1.6) activity was measured based on the decomposition of H_2O_2 (ε= 39.4 mM^{-1} cm^{-1}) at 240 nm for three minutes, using a method developed by Aebi [10]. Each 3 mL reaction solution contained 10 mM H_2O_2, 50 mM K-phosphate buffer (pH = 7.0), 100 µL of enzyme extract. One unit of enzymatic activity was regarded as the amount of CAT that decomposed 1 µmol of H_2O_2 per minute.

Peroxidase (POD) (EC 1.11.1.7) activity was assayed by monitoring the oxidation rate of guaiacol to tetraguaicol (extinction coefficient: 26.6 mM^{-1} cm^{-1}) in a total volume of 3 mL, using a method developed by Kenten and Mann [11]. The reaction mixture included 50 mM potassium phosphate buffer (pH = 7.0), 0.4% H_2O_2, 1% guaiacol and 100 µL of enzyme extract. The increase in absorbance was determined at 470 nm after the augment of H_2O_2 for 10 minutes.

Malonaldehyde (MDA), a final product of lipid peroxidation, was calculated by measuring the concentration of the thiobarbituric acid reactive substances (TBARS), using a method developed by Hodges et al. [12]. 0.3 g leaf tissues were ground and homogenized with 5 mL 10% trichloroacetic acid (TCA), and then transferred into a 10 mL centrifuge tube. After centrifugation (6000 rpm), 2 mL of supernatant was added to 0.6% thiobarbituric acid (TBA). After heating on the boiling water bath for 30 minutes, the compound was immediately cooled in ice water, and then centrifuged (10000 rpm) for 10 min. The TBARS content was computed according to the following equation:

$$\mathrm{TBARS}(\mathrm{nmol}\ g^{-1}\ \mathrm{FW}) = [6.452 \times (A_{532} - A_{600}) - (0.559 \times A_{450}) \qquad (1)$$

Root activity was measured according to 2,3,5- triphenyl tetrazolium chloride (TTC) reduction as described previously [13, 14]. About 0.5 g of root samples were cut into 0.5-1cm sections and

incubated in a mixture solution of 5 ml 1% (w/v) TTC and 5 ml 0.1M phosphate buffer (pH=7.5) at 37 °C for 1 h. The incubation was terminated by adding 1ml 1M sulfuric acid to the mixture. For triphenyl formazan (TF, red product) extraction, the roots were removed and blotted up on filter paper and grinded in the mortar containing 3-5 ml ethyl acetate and a little bit SiO_2 powder. The liquid phase was transferred to a 10 ml stoppered colorimetric tube. Ethyl acetate was added up to the 10 ml level, and the released TF was quantified spectrophotometrically at 485 nm. The concentration of TF reduction was calculated and determine the root vigor as μg $TF \cdot g^{-1}$ $root \cdot h^{-1}$.

Text S9. Analysis of major elemental in plant

In order to monitor the content of P in plant, plant samples were harvested from the each CW reactor and washed thoroughly with deionized water after the long-tern exposure experiments. Roots, stems and leaves were separated and dried off and weighed. The dry samples were ground to fine powders and screened with 100 mesh, and 5 g powder simples were digested with a mixture of HNO_3 and $HClO_3$ (1:1) at 120 °C for 3 h on a heating plate, and the digestion solution after filtrating volume to 20 ml. The concentration of P within the filtrate was analyzed by inductively coupled plasma-optical mass spectrometry (ICP-MS) [15, 16]. The contents of P within every tissue were calculated by the concentration of P and the weight of powder samples.

In order to monitor the content of C, N, H and S in the plant of CWs, plant samples were harvested from the each constructed wetland and washed thoroughly with deionized water after the long-tern exposure experiments. Plants were dried off and weighed. The dry samples were ground to fine powders and screened with 100 mesh [17], then weight 5mg to measure by elemental analyzer (Vario EL cube, Germany).

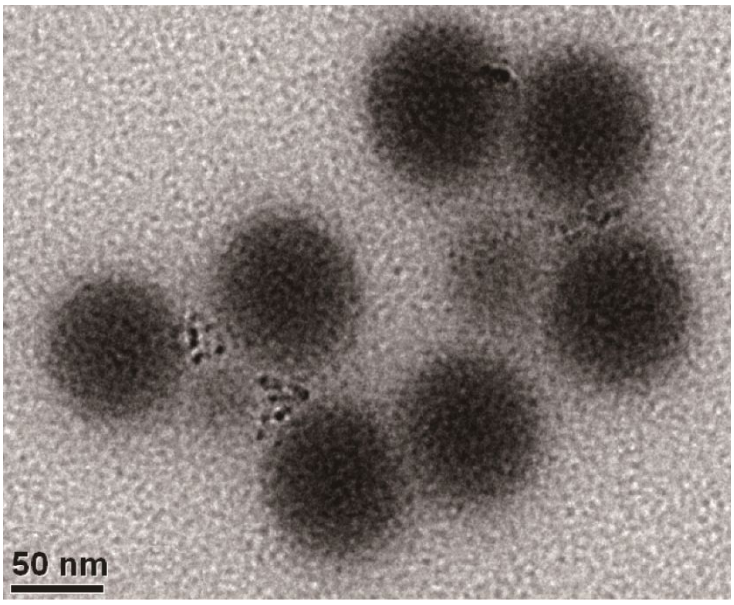

Figure S1. The size distribution images of PS in raw suspension and synthetic wastewater by transmission electron microscopy.

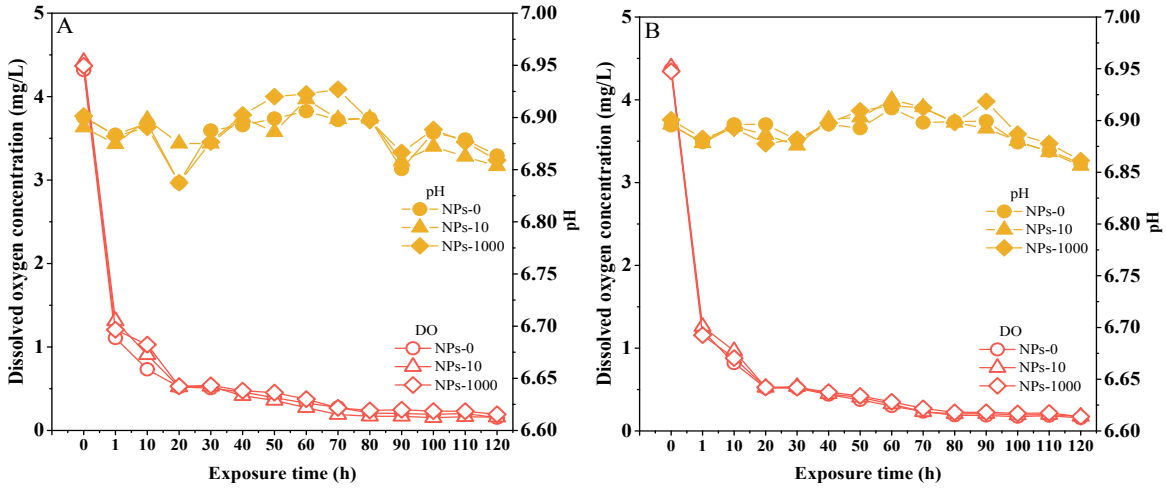

Figure S2. Variations in DO and pH during the first (A) and last (B) batch exposure to PS at different concentrations.

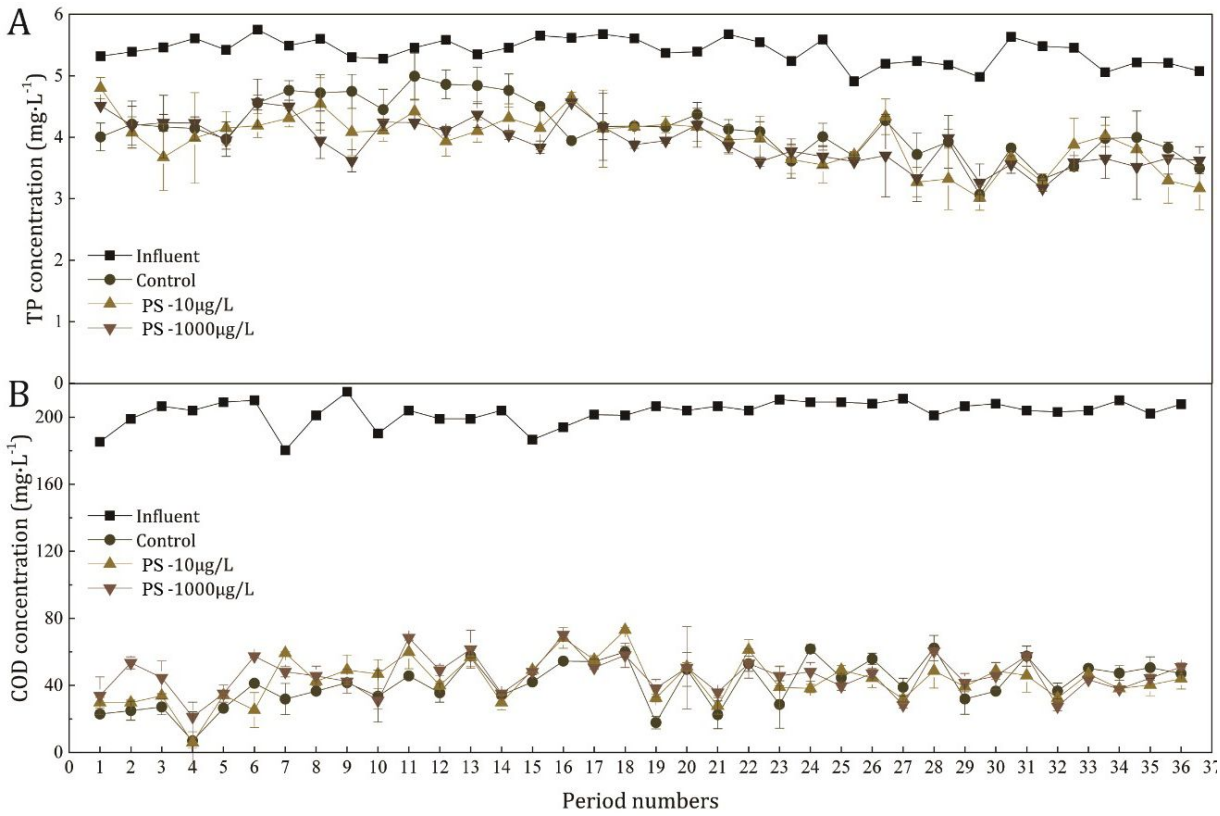

Figure S3. Variations in TP (A) and COD (B) during 180-day exposure to different PS concentrations. All the standard deviations of triplicate measurements are < 10%.

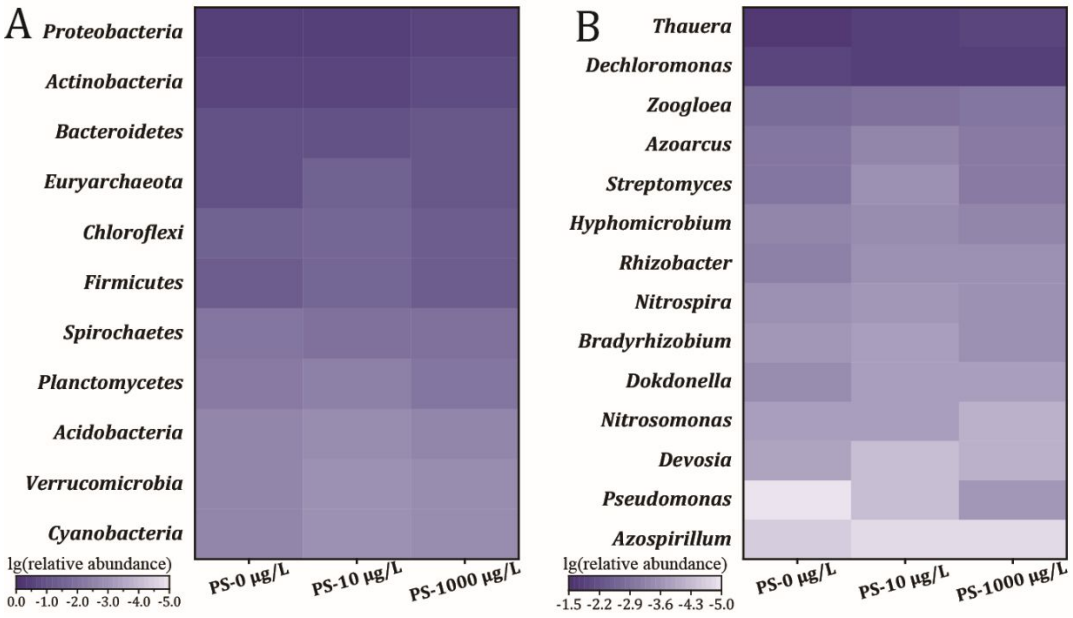

Figure S4. Abundance of the major bacterial phylum (A) and genera (B) levels under the different treatment in the gravel samples collected from CWs treating wastewater containing different concentrations of PS nanoplastics (0, 10 and 1000 μg/L). Colour intensity in each panel shows the relative abundance in one sample.

Table S1. Primers for targeting the denitrifying functional genes in this study .

Genes	Primers
*amo*A	amoA-1FGGGGTTTCTACTGGTGGT
	amoA-2RCCCCTCKGSAAAGCCTTCTTC
*nar*G	narGF TCGCCSATYCCGGCSATGTC
	narGR GAGTTGTACCAGTCRGCSGAYTCSG
*nir*K	F1aCu ATCATGGTSCTGCCGCG
	R3Cu GCCTCGATCAGRTTGTGGTT
*nir*S	Cd3aF AACGYSAAGGARACSGG
	R3cd GASTTCGGRTGSGTCTTSAYGAA

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