Supplementary Materials for

Development of a novel palm fiber biofilm electrode reactor (PBER) for nitrate-contaminated wastewater treatment: performance and mechanism

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1. Electron Transport System Activity (ETSA) analysis

0.5 mL mixed sample of biofilm and solution were collected at 0, 4, 8, 12 and 24 hours at 0, 100, 250 and 400 mA/m^2, and 100 μL INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) (0.5%) was added into sample. The mixture was subsequently incubated at 25 °C for 20 min in the dark, after which 100 μL of formaldehyde were added to terminate the reaction. The samples were subsequently centrifuged at 10000g for 5 min, discarded the supernatant to collect the cells. Next, 2 mL of 96% methanol were used to extract the INF from the bacteria twice, after which the mixed INF extract was measured spectrophotometrically at 490 nm against a solvent blank.[1] The ETSA values were calculated according to the following formula:

$$ESTA\ (\mu g\ O_2\ g^{-1} min^{-1} mg\ protein^{-1}) = \frac{Ab \times V \times 32}{15.9 \times 2 \times S \times t \times m}$$

where, Ab is the sample absorbance, 15.9 is the specific absorptivity of INT-formazan, S and V is the initial volume of sample and the total volume of methanol (mL), t is the incubation time (min), 32/2 is the constant for transformation of μmol INT-formazan to μg O_2, m is the protein concentration of the bacteria (mg protein/mL).

2. EPS extraction and analysis

At the end of the experiment, 15 mL biofilm samples at 0, 100, 250 and 400 mA/m^2 were collected from PBER. Sample was directly centrifuged at 2000 g for 15 min, and the supernatant was discarded. The residual was re-suspended to its original volume using a buffer solution with a pH of 7, consisting of 1.3 mM Na_3PO_4, 2.7 mM NaH_2PO_4, 6 mM

NaCl, and 0.7 mM KCl. The suspension was centrifuged again at 5000 g for 15 min, and the supernatant was collected as the LB-EPS. Then, the residual was re-suspended again with a buffer solution to the original volume; the solution was then treated using ultrasound at 20 kHz and 480 W for 2 min. The extracted solution was centrifuged at 20,000 g for 20 min, and the supernatant was collected as the TB-EPS.[2] Protein (PN) and polysaccharose (PC) were measured using BSA protein quantifying kit (Beyotime Biotechnology, China) and Phenol-Sulfuric acid method, respectively. The EPS extracts of all samples were measured using a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan). MLSS, VSS were measured using standard methods.[3]

Fluorescence excitation emission matrix (EEM) was recorded using a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan). The slit width was 10 nm for both excitation and emission, and the scan speed was set at 40 nm s^{-1}. Excitation and emission were simultaneously scanned at wavelengths ranging from 200 to 450 nm and from 280 to 550 nm, respectively. After regulating the scattering using interpolation in the areas affected by Ravleigh and Raman scatter,[4] the fluorescence regional integration (FRI) technique was adopted for analysis.[5] In order to calculate the total fluorescence, fluorescence excitation/emission boundaries for integration were defined and summarized in Table S1. The regional integrated fluorescence intensity can be calculated using the Eq. S1.[6]

$$\Phi_i = \sum ex \sum em\, I(\lambda ex, \lambda em)\, d\lambda ex\, d\lambda em \quad (S1)$$

where Φ_i is the fluorescence volume beneath each region *i* of EEM; λex and λem indicate the excitation and emission wavelengths in the EEM region.

Table S1 EEM boundaries of the five operationally-defined fluorescence regions.[6]

Type	Region I	Region II	Region III	Region IV	Region V
Ex (nm)	200-250	200-250	200-250	250-400	250-400
Em (nm)	280-330	330-380	380-550	280-380	380-550
Remarks	Tyrosine-like aromatic protein	Tryptophan-like aromatic protein	Fulvic acid-like substances	Soluble microbial byproduct-like substances	Humic acid-like substances

3. The potentiodynamic polarization curve

The potentiodynamic polarization curve was performed on a computer-controlled CHI-660 electrochemical workstation (Chenhua Company, China) using a three-electrode cell (the working electrode was bioelectrode/abiotic electrode, the counter electrode was a platinum electrode, and a saturated mercury chloride electrode was used as the reference electrode). The potentiodynamic polarization curve was conducted between -2.0 V and 0.2 V at scan rate of 10 mV/s.

4. Analysis of palm fiber

As the agricultural and forestry waste, palm fiber contained elements such as C, N and H, so it might serve as the solid carbon source in denitrification. Therefore, palm fiber (1.5 g), $NaNO_3$ (0.304g) and KH_2PO_4 (0.044 g) were added into 1 L deionized water, and 10 mL domesticated denitrifying sludge with 50% sedimentation ratio was also added to carry out denitrification experiments.

To evaluate the changes of element content of palm fibers in PBER, the element analysis of palm fibers was carried out. The fresh palm fibers and the palm fibers after the reaction (225th hours) were washed three times with deionized water and dried in air

for 72 hours. And then cut palm fibers into 1.0 to 2.0 mm. The content of C, H and N in palm fiber was determined by elemental analyzer (Flash 2000, Thermo Fisher, Italy).

Scanning electron microscope (SEM) was performed to investigate the palm fibers changes in surface morphological structure and microbial adhesion. At the end of experiment (225th hours), the solid matrix samples were collected from PBER. The samples and fresh palm fibers were washed gently with a saline (0.9% NaCl, v/v), fixed with 2.5% glutaraldehyde for 4 h, and washed gently 3 times with a phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4). The samples were dehydrated using sequential ethanol concentrations of 30%, 50%, 75%, 90% and 100%, and exposure for 15-20 min per concentration. Then the isoamyl acetate was added to samples to replace the ethanol 2 times for 20 min. The samples were freezed at -20°C for 12 h and air drying at 40°C for 4 h with electric thermostatic drying oven (101-2AB, Tianjin Taisite Instrument Inc., Tianjin, China). Finally, the samples were treated by sputter coating with gold in an ion coater (E-1010, Hitachi, Japan), and then examined with a SEM (SSX-550, Shimadzu, Japan).

5. Microbial diversity analysis

5.1 DNA extraction and PCR amplification

At the end of the experiment, biofilms of no current (0 mA/m^2), the optimal current density (250 mA/m^2) and the original (before inoculate into PBER) were collected to evaluate microbial diversity. Microbial DNA was extracted from these samples using an

E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) using manufacturer protocols. The V4-V5 region of the bacteria 16S ribosomal RNA gene were amplified using PCR（95°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min）using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3) and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

5.2 Illumina MiSeq sequencing

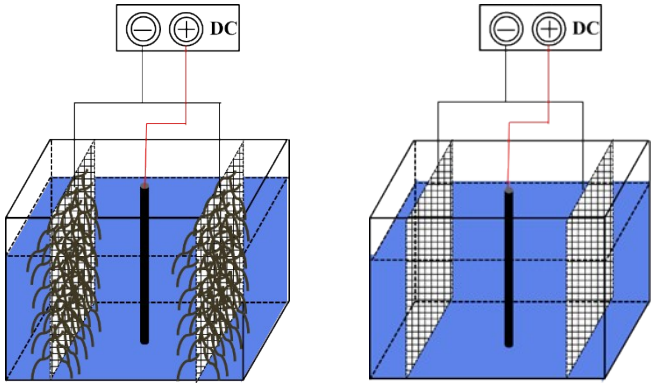
Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

5.3 Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered using QIIME with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than

50bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded.

Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARSE and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier against the silva (SSU115)16S rRNA database using confidence threshold of 70%.[7]



(a) (b)

Fig. S1. The experimental devices of PBER (a) and ER (b).

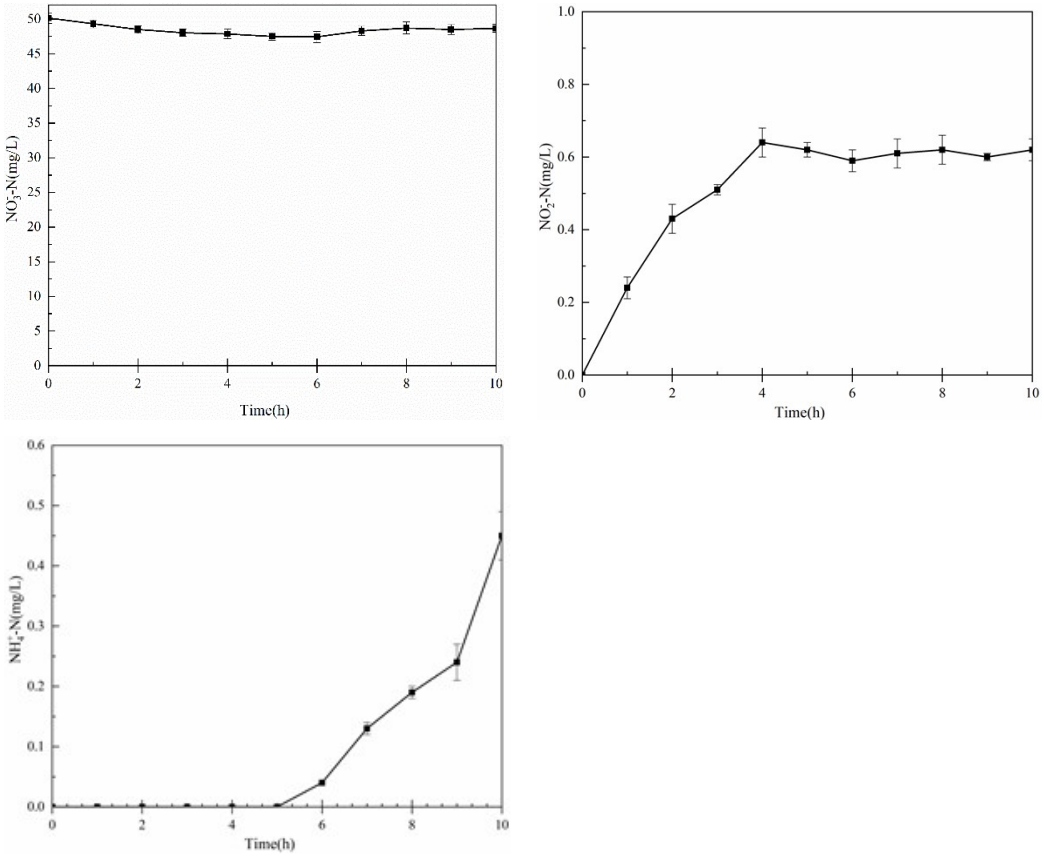


Fig. S2. Denitrification performance of palm fiber as carbon source

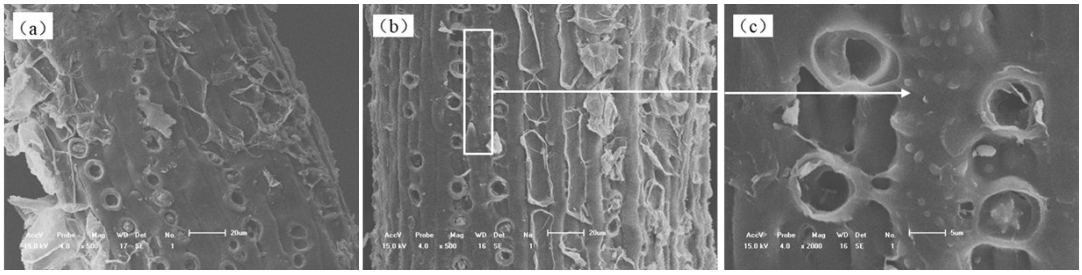


Fig. S3. SEM of palm fiber in PBER: fresh palm fiber (a), palm fiber after reaction (b, c).

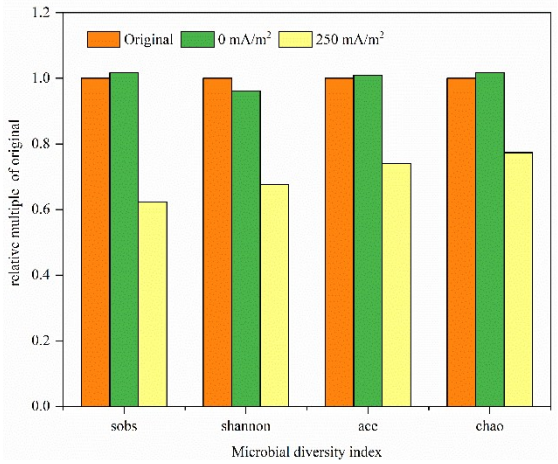


Fig. S4 Relative multiples of microbial diversity in PBER to original sludge at 0 and 250 mA/m^2

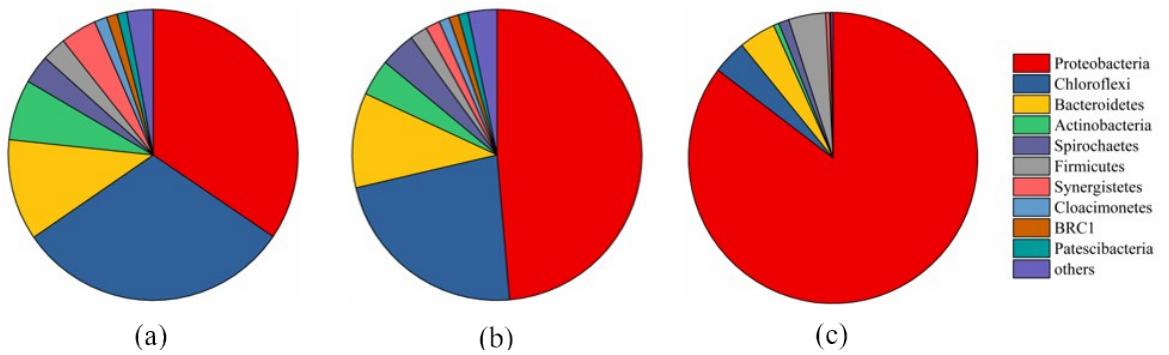


Fig. S5. Microbial community in PBER at phylum level: original (a), 0 mA/m^2 (b), 250 mA/m^2 (c)

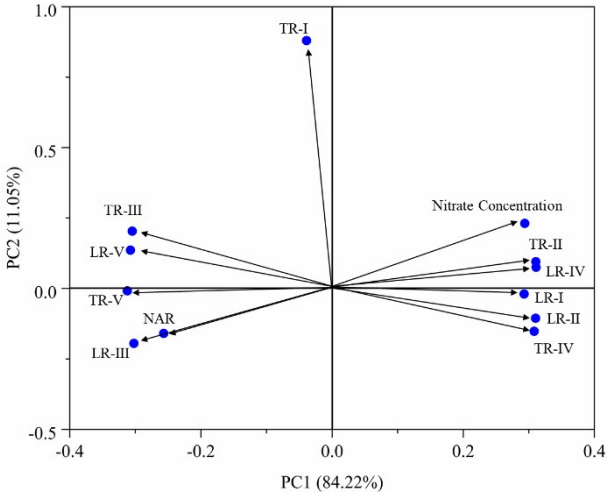
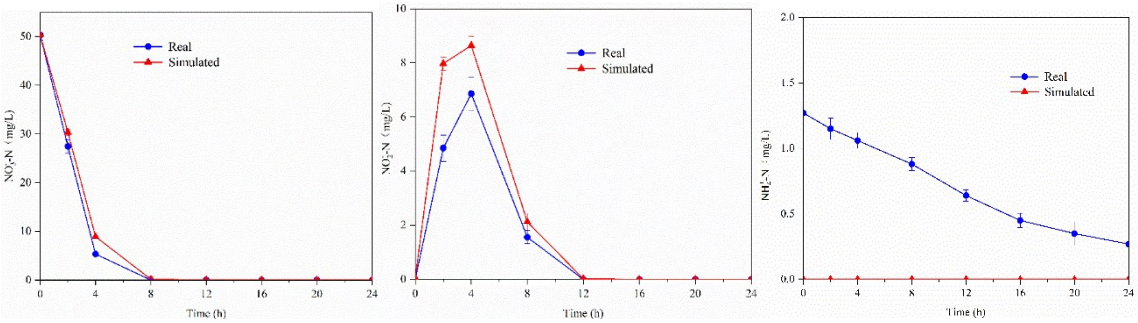


Fig. S6. The principal component analysis of nitrate concentration, NAR and each region of EPS



3 2

Fig. S7. Changes in NO -N, NO -N and NH+ 4-N in PBER treated with simulated real nitrate contaminated water

The groundwater was sampled from a water source reserve well in China University of Geosciences (Beijing, China). Ca^{2+}, Fe^{2+}, Mg^{2+}, Na^{2+} and Mn^{2+} were determined using the inductive coupled plasma emission spectrometer (ICP, iCAP6000, Thermo, US). Cl^- and SO_4^{2-} were measured using an ion chromatograph (ICS 900 Dionex IonPac, Thermo Fisher Scientific, US). The main components of the actual groundwater were 7.04 mg/L NO- 3-N, 92.96 mg/L of Cl^-, 0 mg/L total organic carbon (TOC), 56.22 mg/L inorganic carbon (IC), 1.27 mg/L of NH+ 4-N, 92.86 mg/L of SO_4^{2-}, 84.78 mg/L of Ca^{2+}, 1.50 mg/L of Fe^{2+}, 38.46 mg/L of Mg^{2+}, 26.45 mg/L of Na^{2+}, 0.49 mg/L of Mn^{2+}. Potassium nitrate and sodium acetate were added in the actual groundwater to final concentrations of 50 mg-N/L and C/N of 1.5.

As shown in Fig. S7, concentration of NO- 3-N and NO- 2-N in the real nitrate contaminated water were lower than that in the simulated nitrate contaminated water, which might be attributed to that trace elements in the real groundwater promoted the activity of denitrifying bacteria, nitrate reductase and nitrite reductase.[8] In addition, the

slight decrease in NH+ 4-N concentration might be due to the adsorption of cations by EPS.[9] Therefore, this showed that PBER could be used for nitrate treatment of actual groundwater.

Table S2. Concentration of dissolved hydrogen, TOC and IC at different current densities.

Current density mA/m^2	Dissolved hydrogen mg/L		TOC mg/L		IC mg/L	
	PBER	ER	PBER	ER	PBER	ER
0	-	-	12.51±0.35	-	56.34±3.42	-
100	0.14±0.02	0.32±0.04	30.37±2.14	-	55.56±4.52	21.12±1.02
250	0.65±0.15	1.46±0.21	39.80±1.52	-	55.38±3.87	26.46±1.31
400	0.96±0.22	1.62±0.27	31.24±1.89	-	68.37±3.03	42.98±2.48

Table S3. The diversity index of microbial community at the original, 0 and 250 mA/m^2.

Sample	sobs	shannon	ace	chao	coverage
original	475	4.12	483.34	486.04	0.999334
0 mA/m^2	483	3.96	488.14	494.40	0.999524
250 mA/m^2	296	2.79	358.07	375.88	0.997548

Table. S4 The correlations among nitrate concentration, NAR and different EPS components

	Nitrate	NAR	LR-Ⅰ	LR-Ⅱ	LR-Ⅲ	LR-Ⅳ	LR-Ⅴ	TR-Ⅰ	TR-Ⅱ	TR-Ⅲ	TR-Ⅳ	TR-Ⅴ
Nitrate	1.000	-0.917*	0.788	0.906*	-0.958*	0.939*	-0.855	0.099	0.947*	-0.850	0.874	-0.927*
NAR		1.000	-0.604	-0.787	0.778	-0.785	0.736	0.006	-0.828	0.767	-0.748	0.776
LR1			1.000	0.918*	-0.900*	0.948*	-0.961**	-0.081	0.940*	-0.918*	0.930*	-0.947*
LR2				1.000	-0.939*	0.976**	-0.986**	-0.242	0.967**	-0.987**	0.997**	-0.989**
LR3					1.000	-0.985**	0.911*	-0.098	-0.973**	.881*	-0.921*	0.974**
LR4						1.000	-0.968**	-0.030	0.994**	-0.945*	0.967**	-0.997**
LR5							1.000	0.253	-0.964**	0.992**	-0.992**	0.980**
TR1								1.000	-0.014	0.342	-0.283	0.106
TR2									1.000	-0.944*	0.956*	-0.988**
TR3										1.000	-0.992**	0.964**
TR4											1.000	-0.984**
TR5												1.000

"*" means significant correlation (P<0.05); "**" means highly significant correlation (P<0.01)

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