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The changes of bacterial communities and antibiotic resistance genes in microbial fuel cells during long-term oxytetracycline processing

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ABSTRACT

Microbial fuel cell (MFC) is regarded as a promising alternative for enhancing the removal of antibiotic pollutants. In this study, oxytetracycline served as an electron donor in the anode chamber of MFCs, and after continuous operation for 330 days, the efficiency of removal of 10 mg/L oxytetracycline in MFCs increased to 99.00% in 78 h, whereas removal efficiency of only 58.26% was achieved in microbial controls. Compared to microbial controls, higher ATP concentration and persistent electrical stimulation mainly contributed to bioelectrochemical reactions more rapidly to enhance oxytetracycline removal in MFCs. In addition, the analysis of bacterial communities revealed that *Eubacterium* spp.—as the main functional bacterial genus responsible for oxytetracycline biodegradation—flourished starting from merely 0.00%–91.69% ± 0.27% (mean ± SD) in MFCs. High-throughput quantitative PCR showed that the normalized copy numbers of total antibiotic resistance genes (ARGs) and mobile genetic elements in MFCs were 1.7364 and 0.0065 copies/cell respectively, which were markedly lower than those in the microbial controls. Furthermore, there was no significant correlation between oxytetracycline concentration in the influent and abundance of ARGs in effluent from MFCs. Nevertheless, *Tp614*, a transposase gene, was found to be enriched in both MFCs and microbial reactors, suggesting that it may be a common challenge for different biological processes for wastewater treatment. This study therefore showed a lower probability of upregulation and transmission of ARGs in MFCs when compared to a traditional anaerobic microbial treatment.

1. Introduction

With rapidly rising demand for antibiotics and their overuse in human medicine and animal husbandry, these compounds are regarded as emerging pollutants and were increasingly drawing worldwide attention in recent years (Hirsch et al., 1999; Kümmerer, 2009; Van Boeckel et al., 2015). As one of the most important broad-spectrum antibiotics, oxytetracycline has entered environmental matrices owing to its overuse or abuse, and its residual amounts are exacerbating the emergence of antibiotic resistance genes (ARGs) and superbugs (Li et al., 2008; Liu et al., 2016b). Furthermore, most of traditional treatments cannot remove oxytetracycline efficiently because of its high chemical stability (Liu et al., 2016a; Ternes et al., 2002; Watkinson et al., 2007). Hence, the development of more effective processes for oxytetracycline removal is a hot topic in the field of environmental research (Supporting Information).

Bioelectrochemical systems are considered deeply mineralized and energy recovery devices enhancing the treatment for removal of sulfonamides, nitroimidazoles, and β-lactam and chloramphenicol antibiotics (Guo et al., 2017; Harnisch et al., 2013; Kong et al., 2017; Wang et al., 2015; Wen et al., 2011a, 2011b; Zhang et al., 2016, 2017). Wang et al. achieved highly mineralized removal of 20 mg/L sulfamethoxazole and its byproduct 3-amino-5-

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methylisoxazole in 48 h using bioelectrochemical systems involving an oxidation reaction (Wang et al., 2016). Liang et al. showed that a biocathode in bioelectrochemical systems converted 96.0% ± 0.9% (mean ± SD) of 32 mg/L chloramphenicol via reduction reactions (Liang et al., 2013). Compared to a sequencing batch biofilm reactor, bioelectrochemical systems reduced the abundance of three β-lactam ARGs, i.e., *OXA-1*, *OXA-2* and *OXA-10* along with removal of 90% of cefuroxime (Cheng et al., 2016). These studies have focused mainly on removal efficiency toward antibiotics, but only a small number of ARGs have been analyzed by conventional quantitative PCR. Nevertheless, ARGs as contaminants of emerging concern should have received more attention during these biological treatments, especially during long-term operation. Positive correlations have been found between different ARGs and between ARGs and mobile genetic elements such as plasmids, integrons, and transposons (Zhang et al., 2011; Zhu et al., 2013). Hence, the research on ARGs should not be limited to individual ARGs relevant to antibiotics. With the aid of high-throughput quantitative PCR (HT-qPCR), a deep insight into the dissemination of ARGs has been acquired (Hu et al., 2017; Xie et al., 2016; Xu et al., 2016; Zhu et al., 2017). To better assess whether the bioelectrochemical system is a promising alternative for enhancing antibiotic removal in terms of the fate of ARGs, it is important to investigate the fate of a board range of ARGs under different operating conditions.

In the present study, microbial fuel cell (MFC) as one of typical bioelectrochemical systems was chosen, and high-throughput sequencing and HT-qPCR were employed to investigate the changes in bacterial communities and ARG profiles in MFCs during long-term oxytetracycline degradation. The main objectives of this study were (1) to characterize the dynamics of oxytetracycline removal in MFCs during 330-day operation, (2) to reveal the succession of the bacterial community during the long-term processing of oxytetracycline, (3) to investigate the effects of oxytetracycline on ARGs in biofilms and effluents. These findings can systematically elucidate the biodegradation of oxytetracycline in MFCs and the fate of ARGs during long-term treatment of oxytetracycline-contaminated wastewater.

2. Materials and methods

2.1. Chemicals and analytical methods

Oxytetracycline (>90%) was purchased from Aladdin Industrial Corporation (Shanghai, China), and an oxytetracycline standard (as hydrochloride) suitable for high-performance liquid chromatography (HPLC) analysis was ordered from Dr. Ehrenstorfer (GmbH, Augsburg, Germany). Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). All the other chemicals (analytical grade) were bought from Sinopharm Group Co., Ltd. (Shanghai, China). Oxytetracycline dissolved in methanol (1 mg/mL) served as a stock solution, which was stored at −20 °C and was replaced once a month.

2.2. Reactor setup

Nine two-chamber MFCs with 140 mL working volume of each chamber were constructed by assembling acrylic glass plates into a hollow rectangular block (7.0 × 5.0 × 4.0 cm). The MFCs were constructed according to our previous studies (Wang et al., 2016; Xiao et al., 2013, 2016). A cation exchange membrane (Zhejiang Qianqiu Water Treatment Co., Ltd., China) was used to separate the anode and cathode chamber. The anodes and cathodes were made of carbon felts (4 × 4 × 0.5 cm, Haoshi Carbon Fiber Co., Ltd., China), which were pretreated by steeping in acetone for 48 h and then immersing in deionized water for 24 h. Titanium wire (1 mm in diameter) was employed to connect the electrodes, and the external load was a resistor of 500 Ω, but the anode and cathode were disconnected when they served as microbial controls, i.e., MFCs in an open-circuit state. The output voltage values of MFCs were recorded by a digital multimeter (Keithley Instruments, Inc., USA). The electrodes in abiotic controls were autoclaved.

2.3. Reactor operation

For comparison, three different rectors were operated as follows: MFCs with a closed circuit (MFC), MFCs in an open-circuit state (microbial control), and MFCs with an abiotic anode (abiotic control; Supporting Information Fig. S1). To construct biofilms of MFCs and microbial controls, the anode chambers of six MFCs were inoculated with a mixture of supernatants from pig manure (LeSen Farm, Xiamen, China) and artificial wastewater at a ratio of 1:5 (v/v). The artificial wastewater thereafter served as an anolyte and contained a buffering solution (1 g/L sodium acetate and 50 mmoL/L phosphate, pH 7.0) (Lovley and Phillips, 1988; Xiao et al., 2013). The catholyte contained 100 mmoL/L $K_3[Fe(CN)_6]$ and 50 mmoL/L phosphate buffer solution (pH = 7.0). Both the anolyte and catholyte were refreshed biweekly in batch mode, and the maximum voltage reached 0.6–0.7 V after approximately 1 month, when the MFCs were regarded as successfully developed. Then, oxytetracycline served as a carbon source instead of sodium acetate in the anolyte, and its concentration has been gradually increased from 0.5 to 10 mg/L for the next 10 months. All the reactors were kept in a dark incubator (LRH-500F, Keerlrein instrument Co., Ltd., Shanghai, China) to avoid photodegradation of oxytetracycline.

2.4. Analytical methods

The analysis of high oxytetracycline concentrations was accomplished by means of a Hitachi L-2000 series HPLC system (Hitachi, Japan) equipped with a diode array detector and column oven. An Agilent column (Zorbax Eclipse Plus C18, 4.6 × 250 mm, 5 μm) was applied to separate oxytetracycline, and the temperature of the column was set to 30 °C. The injection volume was 10 μL, and the diode array detector detection was set to 278 nm. The mobile phases were 0.01 moL/L oxalic acid in Milli-Q water (A), acetonitrile (B), and methanol (C) (72:18:10, v/v/v). Isocratic elution at a flow rate of 1.0 mL/min was maintained for 8 min. All the solvents for HPLC were passed through a filter (0.45 μm pore size) and ultrasonicated for 30 min for degassing. All effluent samples were filtered through a membrane with 0.22 μm pore size before use.

Low oxytetracycline concentrations (<1 mg/L) were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS; an ABI 3200 Q TRAP instrument, USA) as described previously with a modification (Ashfaq et al., 2017; Sun et al., 2016). Each supernatant from MFCs and microbial controls was passed through 0.22 μm filters, diluted with Milli-Q water, then adjusted to pH 2.0, and finally processed in solid-phase extraction cartridges (Oasis HLB, 60 mg/3 cc, Waters, Milliford, MA). Oxytetracycline separation was performed on a Kinetex C18 column (4.6 × 100 mm, 2.6 μm, Phenomenex, CA, USA) on an LC system (Shimadzu, Japan). The mobile phase consisted of 0.1% formic acid (A) and methanol (B). A binary gradient at a flow rate of 0.5 mL/min was implemented as follows: the level of solvent A decreased gradually from 85% to 80% for 1 min and continued to decrease to 70% for 3 min, then decreased to 15% in 3–6.5 min, and finally returned to the initial setting in 9 min. The mass measurements were performed by means of an ABI triple-quadrupole (QqQ) mass spectrometer. The declustering potentials, entrance potentials, collision energies, and collision cell exit potentials were set to 45.00, 8.00, 25.00, and 17.00 eV, respectively.

2.5. Sample collection and DNA extraction

The biofilm samples of MFCs and of microbial controls were collected at the following stages: (1) raw pig manure was collected before adding into reactors; (2) sodium acetate feeding stage: sodium acetate as a carbon source during 1-month operation; (3) oxytetracycline feeding stage: oxytetracycline as a carbon source during 10-month operation. The samples from effluents after addition of 0.5, 1, 3, 5, or 10 mg/L oxytetracycline into MFCs and respective microbial controls were collected by centrifuging water samples. All the samples were stored at −80 °C for further analysis.

The FastDNA®Spin Kit for soil (MP Biomedical, Santa Ana, CA, USA) was used to extract total DNA from biofilm samples and from effluent samples. The extracted DNA concentrations were measured by spectrophotometric analysis on a NanoDrop ND-1000 (Nanodrop, USA), and the DNA was stored at −20 °C until use.

2.6. 16S rRNA gene amplification, sequencing, and data processing

To investigate the shifts in bacterial communities, the V3-V4 region of the bacterial 16S rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), purified, quantified, pooled, and sequenced. PCR was conducted on a TransStart FastPfu DNA Polymerase system. The 20 μL PCR mixture was prepared in accordance with our previous studies (Xiao et al., 2016). The PCRs were run on an ABI GeneAmp®9700 PCR instrument with the following parameters: one cycle at 95 °C for 3 min; 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and finally 72 °C for 10 min. The subsequent high-throughput sequencing was performed at Majorbio Bio-Pharm Technology Co., Ltd., (Shanghai, China) on the MiSeq PE platform (Illumina, USA).

Sequences shorter than 20 bp and with a quality score of lower than 30 were removed from the data. Sequences with over 97% identity were clustered into one operational taxonomic unit (OTU) in the Usearch software (version 7.1) (Edgar, 2013). Community richness, Chao1 richness estimates, Ace and Shannon indices, and rarefaction curves were obtained by MOTHUR analysis (Schloss et al., 2011). The taxonomic identities of sequences were assigned on the QIIME platform via the RDP Classifier at a confidence level of 70% based on the Bayesian algorithm (Wang et al., 2007).

2.7. HT-qPCR

This analysis of ARGs was performed to measure the diversity and abundance of ARGs using a SmartChip Real-time PCR machine (Warfergen Inc., USA) as described elsewhere with a modification (Su et al., 2015). A total of 57 primer sets (Table S1) were used including one targeting the 16S rRNA gene, ten targeting mobile genetic elements (containing eight transposase genes and two class I integrase genes), seven targeting sulfonamides genes, and 39 targeting tetracyclines genes. The following thermal cycling conditions were set up: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s, ending with a melting curve analysis automatically generated by the program. For each primer set, amplification was conducted in triplicate, and a nontemplate control was included.

The results of HT-qPCR were analyzed in the SmartChip qPCR software (version 2.7.0.1). Wells with multiple melting peaks were discarded, as well as wells with amplification efficiency outside the range of 1.8–2.2. Threshold cycle (C_T) 31 served as the detection limit, and only samples with three replicates showing successful amplification were regarded as yielding a positive result. The relative copy number was calculated according to another study (Eq. (1)) (Looft et al., 2012). Besides, another method (Eq. (2)) was applied to figure out ARGs' fold changes (FC values) in effluent samples compared to the raw pig manure (Schmittgen and Livak, 2008):

$$\text{Gene Copy Number} = 10^{((31-C_T)/(10/3))} \quad \Delta C_T = C_{T(ARG)} - C_{T(16S)} \tag{1}$$

$$\Delta\Delta C_T = \Delta C_{T(Target)} - \Delta C_{T(Ref)} \quad \text{FC} = 2^{(-\Delta\Delta C_T)} \tag{2}$$

where C_T is the threshold cycle, $C_{T(ARG)}$ corresponds to the threshold cycle of one of the 56 antibiotic resistance gene assays, $C_{T(16S)}$ corresponds to the threshold cycle of the 16S rRNA gene assay, $\Delta C_{T(Target)}$ corresponds to the difference value between the threshold cycle of the experimental sample and the threshold cycle of its 16S rRNA gene assay,$\Delta C_{T(Ref)}$ is the difference value between the threshold cycle of the reference sample and the threshold cycle of its 16S rRNA gene assay, and FC is the fold change of an ARG copy number.

2.8. Statistical analysis

The data of averages, standard deviations, and fold changes of ARGs were organized in Excel 2010 (Microsoft, USA). ARGs were considered statistically significantly enriched or depleted if the range consisting of three standard deviations of the mean fold change was entirely >1.0 or <1.0, respectively (Su et al., 2015). Principal coordinate analysis (PCoA) based on Bray–Curtis distance and a heatmap were generated by R language (version 3.1.0). The linear discriminant analysis (LDA) effect size algorithm (LEfSe) was applied to explore the statistically significantly features of microbial communities between different samples (Segata et al., 2011). Other plots were performed in Origin Pro 9.1 software (OriginLab, USA).

3. Result and discussion

3.1. Removal of oxytetracycline

The changes in oxytetracycline concentration after 1-month acclimation are illustrated in Fig. 1A. No obvious change in oxytetracycline concentration was observed in the abiotic control, suggesting that carbon felt adsorption and oxytetracycline self-degradation were negligible. It was observed that after the 1-month acclimation, the efficiency of removal of 0.6 mg/L oxytetracycline by MFCs in 6 days was nearly 85%: a little higher than that in the microbial control. By contrast, the oxytetracycline removal rate increased obviously after 10-month acclimation (Fig. 1B). The removal efficiency rates in the MFCs and in the microbial control were 99.00% and 58.30%, respectively. In contrast, oxytetracycline concentration barely changed in the abiotic controls. Hence, the decrease in oxytetracycline concentration in MFCs and in the microbial controls must be due at least in part to biodegradation, and after long-term acclimation, bioelectrochemical reactions in MFCs apparently enhanced the oxytetracycline removal. The results of the antibacterial assay of the effluents from MFCs by means of *Escherichia coli* DH5α and *Shewanella oneidensis* MR-1 revealed that the antibacterial ability of oxytetracycline parent modules and its metabolites were eliminated successfully after the bioreactor treatment (Fig. S3).

In MFCs, the anode acted as an insoluble electron acceptor to quickly receive electrons generated by anodophilic bacteria. The persistent electrical stimulation, on the one hand, promoted the growth and metabolic activity of exoelectrogenic bacteria that

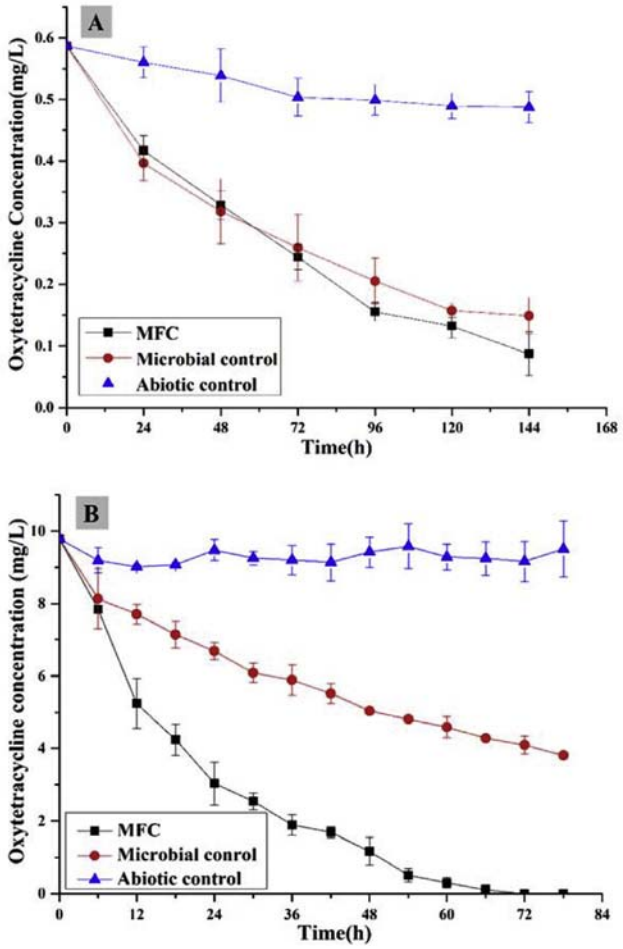

Fig. 1. Oxytetracycline concentration changes during a single cycle in MFCs, in a microbial control (no current), and in an abiotic control (without current and microorganisms) after acclimation to oxytetracycline for 1 month (A) and 10 months (B), respectively. The initial oxytetracycline concentration in each cycle was 0.6 mg/L and 10 mg/L, respectively. All the reactors were operated in batch mode. A "cycle" means that the anolyte and catholyte were refreshed when the response of voltage declined to less than 100 mV.

were involved in oxytetracycline metabolism, and on the other hand, might have made MFCs more suitable niches for the oxytetracycline degradation by increasing the metabolic reaction rates of microbes on the anode. Similar findings have been obtained in other studies (Cao et al., 2015; Zhang et al., 2017). This function did not work in microbial controls, and this is one of the reasons why MFCs showed higher removal efficiency than the microbial controls did.

In addition, voltage output—as an important indicator of the performance of MFCs—was recorded during the 330-day operation (Fig. S2). The voltage decreased sharply with oxytetracycline substitution for acetate as a substrate but gradually recovered during the acclimation process, indicating that anodic microorganisms in MFCs could metabolize oxytetracycline to grow and to yield electricity. With prolongation of the operation, the microbes in MFCs colonized the electrodes tightly, formed a solid network connected by nanowire-like matrices (Fig. S4), and showed higher microbial activity than that seen in microbial controls (Figs. S5 and S6). The results therefore indicated that microbes in MFCs were more active at utilizing oxytetracycline for their reproduction than those in microbial controls.

3.2. Characterization of bacterial communities

After the long-term operation, the succession of microbes played an indispensable role in enhancing oxytetracycline biodegradation in MFCs. A total of 498,017 high-quality sequences were recovered from all 13 samples, which were clustered into 2674 OTUs at a similarity level of 97.00% (Table S2). The coverage indices and the flat rarefaction curves confirmed that most microbes were covered in this sequencing analysis (Fig. S7). Chao1, Simpson, and Shannon indices suggested that long-term oxytetracycline processing significantly reduced the microbial community richness and diversity, especially in MFCs. These data indicated that the role of anaerobic conditions coupled with electrical stimulation had a more profound selection effect on specializing functional species than that seen in microbial controls only under anaerobic conditions. PCoA indicated that the substrate and operation mode both contributed to the shifts in the bacterial community (Fig. 2), as confirmed by PCoA of weighted and unweighted unifrac distances (Fig. S8).

In-depth analysis of the predominant taxa that illustrates the main differences between MFCs and microbial controls at different acclimation time points was carried out by the LEfSe algorithm (Fig. 3). Judging by statistically significant differences, 32 biomarkers were found at an LDA threshold of 4.0 (Fig. 3B). At the phylum level, an obvious distinction between MFCs and microbial controls at two acclimation time points was observed and confirmed the conclusion about the effect of the carbon source and operation mode on microbial communities. Compared to other samples, Firmicutes as the dominant phylum was found to be greatly enriched (to 94.70%) in oxytetracycline-fed MFCs (Fig. S9). Some studies on the microbial communities in MFCs fed different fermentation end products have shown that Firmicutes play a vital role in the processing of complex substances (Kiely et al., 2011). In addition, our data are in agreement with other studies showing that Firmicutes become the dominant phylum under long-term antibiotic selection pressure (Li et al., 2011; Qiu et al., 2013).

The structure of the microbial communities at the class level manifested variation during the 330-day operation although LEfSe

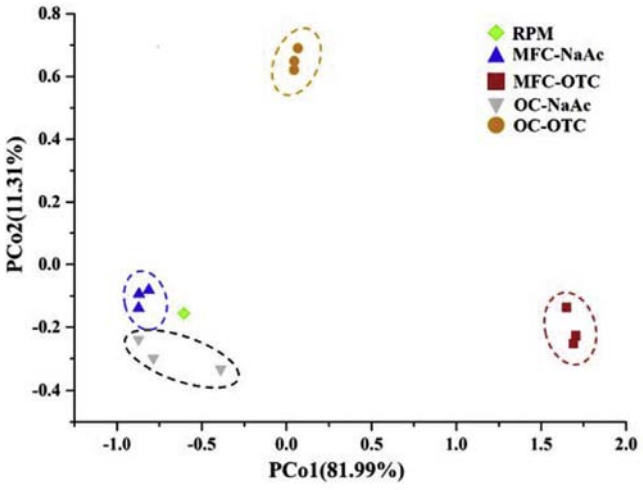

Fig. 2. PCoA based on the Bray-Curtis distance showed the overall distribution pattern of bacterial taxa in the microbial communities. RPM indicates the sample of raw pig manure; MFC-NaAc and MFC-OTC represent the samples of MFCs fed with sodium acetate and oxytetracycline, respectively; OC-NaAc and OC-OTC denote the samples of microbial controls fed with sodium acetate and oxytetracycline, respectively.

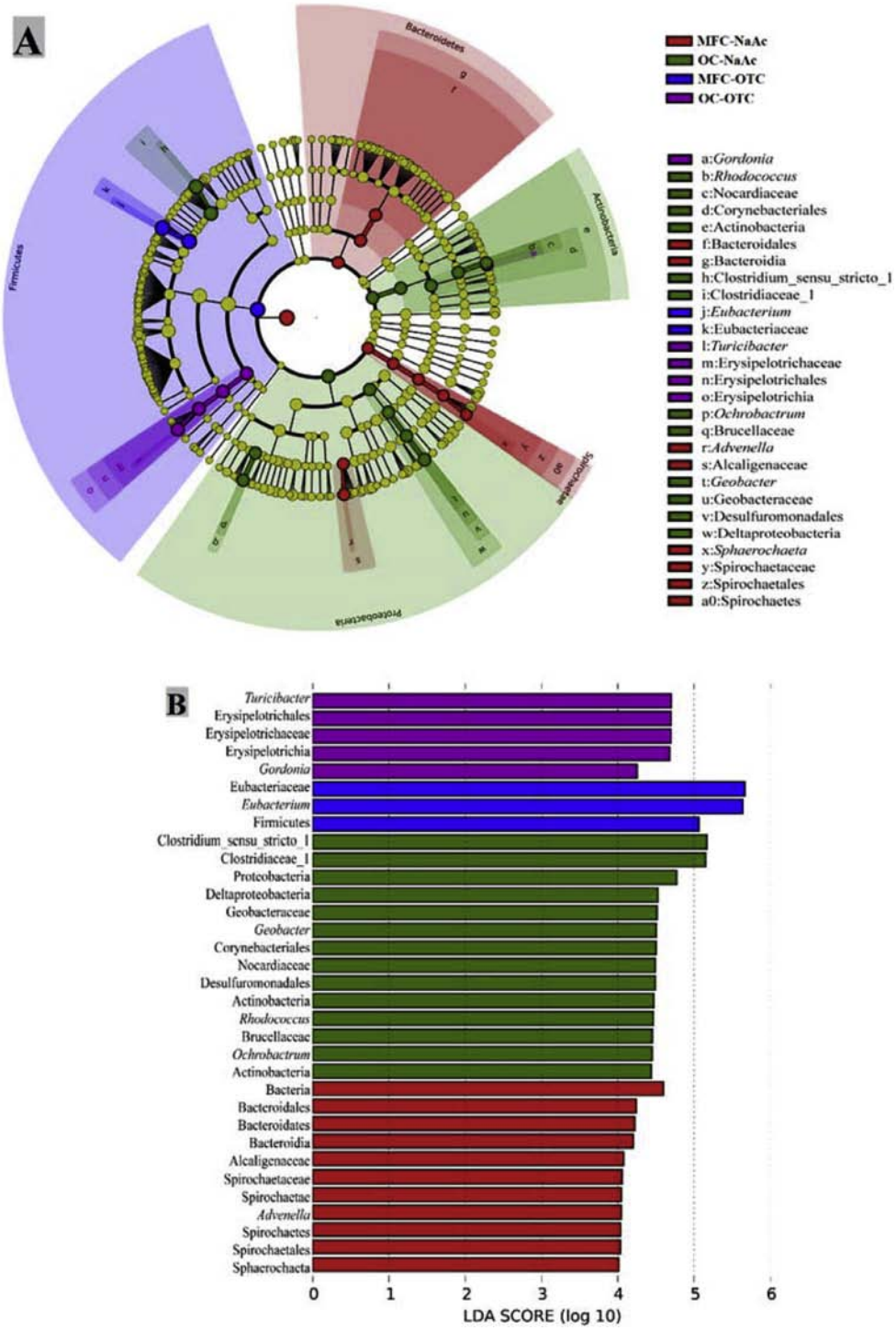

Fig. 3. LEfSe analysis based on the samples of MFCs and microbial controls at sodium acetate and oxytetracycline stages in various abundant taxa. (A) A cladogram of the samples of MFCs and microbial controls at sodium acetate and oxytetracycline stages. (B) The linear discriminant analysis (LDA) score of abundant biomarkers from all samples. MFC-NaAc and MFC-OTC denote the samples of MFCs fed with sodium acetate and oxytetracycline, respectively; OC-NaAc and OC-OTC represent the samples of microbial controls fed with sodium acetate and oxytetracycline, respectively. Differences are represented by colors (red for NaAc-MFC, green for NaAc-OC, blue for OTC-MFC, and fuchsia for OTC-OC) of the most abundant biomarkers, and each circle's diameter is proportional to the relative abundance of taxa. The inner to outer circles correspond to kingdom to genus levels, and taxa with significant differences in all samples are marked by a corresponding color and show a concrete taxon name and LDA score in panel B. The other layers of taxa, which might be important but did not show obvious differences in all samples, are colored yellow in panel A. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analysis did not yield this result. The relative abundance of Clostridia in oxytetracycline-fed MFCs, one of the most strictly anaerobic classes, increased obviously: from 71.0% to 93.6% (Fig. S9). It has been reported that the peak voltage and charge in MFCs were tightly associated with the abundances of Clostridiaceae, Lachnospiraceae and Peptostreptococcaceae all belonging to Clostridia, implying that the Clostridia class played a significant role in power generation in MFCs (Jiang et al., 2016). In addition, many bacteria belonging to Clostridia can degrade a large array of complex carbohydrates to produce a wide range of simple metabolites like ethanol (Elsden et al., 1976; Gu et al., 2014). It was noticeable here that α-, β-, and γ-Proteobacteria in oxytetracycline-fed MFCs, accounting for 1.3%–1.4%, 1.5%–1.6%, and 0.8%–1.6%, respectively, were still not eliminated after a long period of operation. Recent studies on removal of the chloramphenicol antibiotic by a biocathode revealed that α-, β-, and γ-Proteobacteria perform a significant function in the degradation of chloramphenicol (Liang et al., 2013). In addition, research has revealed that bacteria belonging to β- and γ-Proteobacteria dominated in anoxic or aerobic membrane bioreactors processing oxytetracycline, tetracycline, and sulfamethoxazole (Xia et al., 2012).

Next, a comparison of the microbial communities at the genus level was conducted. LEfSe analysis revealed that *Eubacterium* spp. in oxytetracycline-fed MFCs manifested a remarkable difference from other samples. Its relative abundance underwent a noteworthy increase from merely 0.0% in raw pig manure to 91.8% (Fig. S10). An exoelectrogenic bacterium belonging to *Eubacterium* spp. was isolated from soil and the maximum power density of an MFC generated by this isolate was about 19 mW/m^2 (Jiang et al., 2016). These results indicated that this member of *Eubacterium* spp. played a role in electron transfer and power generation in MFCs. Additionally, Qiu et al. have reported that a *Eubacterium* sp. was identified as a functional species during biodegradation of the berberine antibiotic whose molecular structure is similar to that of oxytetracycline (Qiu et al., 2013). Some species of *Eubacterium* have also been reported to be responsible for anaerobically transforming various oxygen-containing heterocyclic and methoxylated aromatic compounds (Mountfort et al., 1988; Zeng et al., 2017). *Eubacterium* sp. strain SDG-2 possess abilities of heterocyclic ring cleavage and *p*-dihydroxylation split in the aromatic rings of catechins (Wang et al., 2000, 2001). *Eubacterium ramulus* is capable of cleaving oxygen-containing heterocyclic ring system of various flavonoids under anaerobic condition (Schneider and Blaut, 2000). Furthermore, some members of *Eubacterium* sp. could secrete enzymes to catalyze metabolic processes, including demethylation, dehydroxylation, ring cleavage and oxidation, of complex compounds. For example, by the action of a four-component *O*-demethylase, *Eubacterium limosum* ZL-II could convert secoisolariciresinol to its demethylating products, which were easily oxidized due to their low redox potentials (Chen et al., 2016). And methyltransferase secreted form *Eubacterium* sp. ARC-2 facilitating the metabolic reaction of demethylating arctigenin was also found (Jin et al., 2007). Besides, phenolic hydroxyl- and methyl-groups as well as heterocyclic aromatic rings are present in the chemical structure of oxytetracycline. Therefore, taken together, the data on quick and efficient antibiotic removal after long-term acclimation and results of the above-mentioned studies suggest that *Eubacterium* is the main functional bacterial genus biodegrading oxytetracycline and its metabolites.

3.3. Variation in ARGs in biofilms during long-term operation

Long-term antibiotic treatment means that microbes are subjected to stressful selective pressure to increase the dissemination of ARGs, which is a common challenge in all biological systems designed for removal of antibiotics (McKinney et al., 2010; Miller et al., 2016). Hence, it is important to evaluate the fate of ARGs in MFCs and in microbial controls during 330-day oxytetracycline processing.

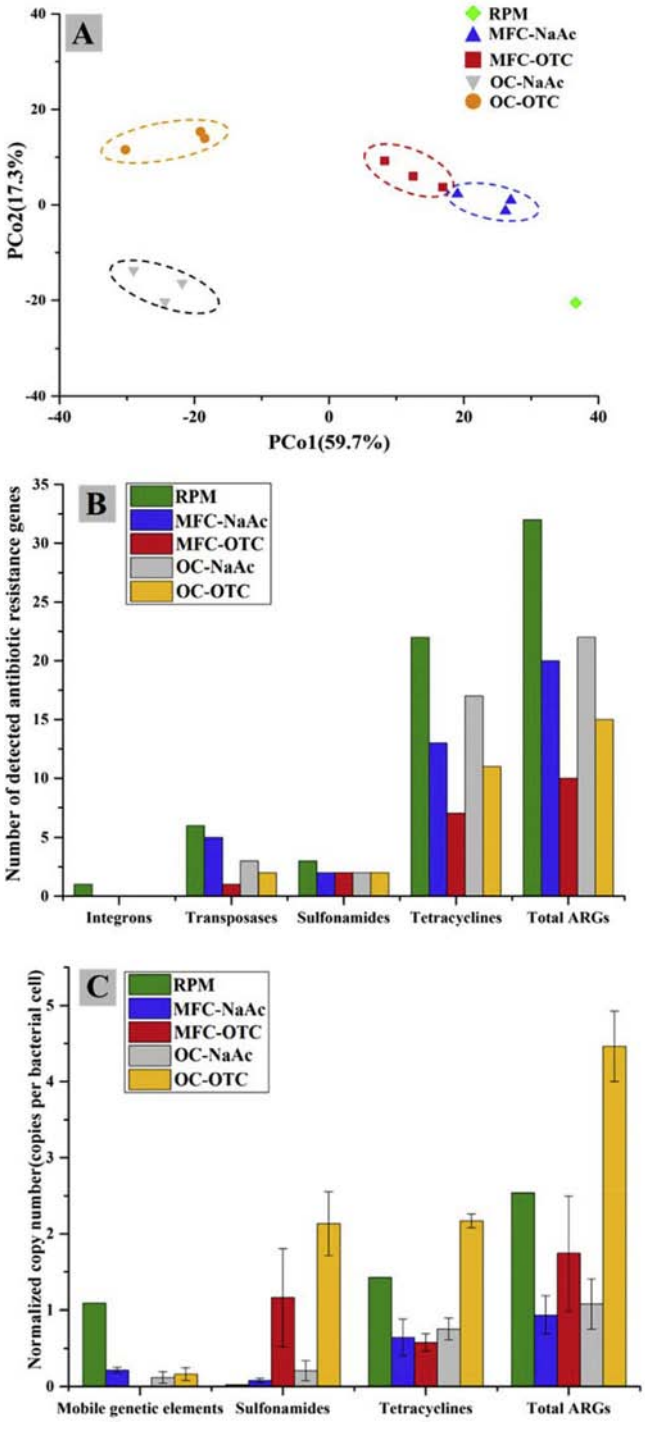

Fig. 4. The overall pattern of ARGs of raw pig mature and biofilms in MFCs and in microbial controls. (A) PCoA based on the Bray-Curtis distance uncovered the overall distribution pattern of the main ARGs in biofilms. (B) The variation of ARG types in all samples. (C) The ARGs' normalized abundance levels in biofilms of MFCs and microbial controls. RPM indicates the sample of raw pig manure; MFC-NaAc and MFC-OTC represent the samples of MFCs fed with sodium acetate and oxytetracycline, respectively; OC-NaAc and OC-OTC denote the samples of microbial controls fed with sodium acetate and oxytetracycline, respectively; Sulfonamides denote the sulfonamide-related ARGs; Tetracyclines denote the tetracycline-related ARGs.

The overall PCoA pattern based on ARGs in all 13 samples is illustrated in Fig. 4A. The samples from the same experimental group could be easily assigned to the same group in the PCoA pattern, indicating good reproducibility of the experiments. PCo1 separated the samples of MFCs from the samples of microbial controls; This finding indicated that the operational mode may be the most influential factor for the ARG profile. The samples from MFCs fed with acetate and oxytetracycline were clustered into two close-knit groups by PCo2 in ARG profiles, whereas the samples of OC-NaAc and OC-OTC were separated substantially. These results revealed that the substrate replacement had a big impact on the ARG profile in the traditional anaerobic reactor but barely in MFCs. That is to say, the type of ARGs (i.e., the pattern of ARGs) present in MFCs is more stable.

A total of 56 unique ARGs, mainly conferring resistance to tetracyclines and sulfonamides, were analyzed in all samples. In sample RPM, 32 ARGs were detected, which were assumed to come from inoculation sources and from the total original ARGs (Fig. 4B). These 32 ARGs included six transposase genes and one integron gene regarded as horizontal transfer genes, implying that raw pig manure is a possible reservoir of ARGs and may cause horizontal transfer of ARGs if handled inappropriately (Zhu et al., 2013). Nevertheless, ARG abundance levels in all the reactors sharply decreased as compared to raw pig manure, in particular, the integron gene was not detected no matter what substrates were fed to biofilms. The total ARG types in oxytetracycline-fed MFCs declined to one-third of that in RPM and was also the lowest among all the samples. This result may be mainly related to the noteworthy reduction in the bacterial communities' diversity in these MFCs. In other words, *Eubacterium* spp. as the sole dominant genus in MFCs may not acquire or upregulate various types of ARGs under oxytetracycline pressure (Fig. S10).

To evaluate relative abundance of ARGs in all the samples, all ARG abundance levels were normalized to the single-cell level (Fig. 4C) (Klappenbach et al., 2001; Stalder et al., 2013). In sample RPM, the normalized copy number of mobile genetic elements exceeded 1.00, and this value for ARGs surpassed 2.50, suggesting that each bacterial cell harbored at least 1.00 mobile genetic element and possessed a certain potential for horizontal gene transfer. The normalized copy numbers of all ARGs from the bacterial communities fed with acetate in both MFCs and in microbial controls were much lower than those of biofilms fed with oxytetracycline. This finding confirmed that the substrate can influence the fate of ARGs, and biological reactors containing antibiotics may facilitate the proliferation of ARGs to some extent. Nonetheless, except for sulfonamide-related ARGs, the normalized copy numbers of the other classes of ARGs in MFCs were much lower than those in RPM, even though anodic biofilms of MFCs were under long-term oxytetracycline pressure. Moreover, the normalized copy numbers of mobile genetic elements and sulfonamide-related and tetracycline-related ARGs in MFCs were 0.0064, 1.16, and 0.57, respectively, which were much lower than those of microbial controls (0.16, 2.13, and 2.16, respectively). This result implies that MFCs may be a more favorable approach to decreasing the dissemination of ARGs in biofilms in comparison with microbial controls representing the traditional anaerobic systems. That is, MFCs may decrease oxytetracycline pressure more effectively by selecting a stand-alone functional microbial taxon more rapidly, thereby reducing the variety of ARGs and their abundance. In addition, it has been reported that anaerobic systems are favorable for the removal of ARGs (Diehl and LaPara, 2010; Pei et al., 2007), and in this regard, MFCs constructed on the basis of anaerobic conditions may be superior to traditional biological processes.

3.4. The changes of ARGs in effluents

The diversity and abundance of ARGs in effluents of MFCs (as a technology for wastewater treatment) has remained obscure to date. To study the effect of different initial oxytetracycline concentrations on the fate of ARGs in effluents, ARG enrichment as compared to sample RPM was next analyzed and presented as a heatmap (Fig. 5).

Among the 56 target ARGs, most ARGs showed extensive depletion, but 16 ARGs, including twelve tetracycline-related ARGs, one sulfonamide-related ARG, and three mobile genetic elements were enriched in all the samples. On the other hand, no clear-cut correlation was found between environmental factors, such as the treatment method or antibiotic concentration, and the enrichment of ARGs. PCoA analysis based on ARG distribution in effluents confirmed this result (Fig. S11), where all samples were distributed randomly aside from grouping in specific clusters by treatment method and by antibiotic concentration.

Of note, not all tetracycline-related ARGs were enriched in effluents under long-term oxytetracycline pressure. Furthermore, ARGs in the same reactor at different concentrations or in different reactors at the same concentration of oxytetracycline did not yield a positive correlation. For example, in the MFC, *tnpA-04* was depleted almost 1000-fold during 5 and 10 mg/L oxytetracycline treatment, but *sul2* was the most depleted gene in the samples corresponding to 0.5 and 3 mg/L oxytetracycline, almost 100-fold and 1000-fold, respectively. Notably, *intI-1* as an integron gene has been reported to be enriched by biological treatments according to conventional qPCR (Yuan et al., 2016) but appears to be depleted in MFCs and microbial controls in this study. By contrast, *Tp614* as a transposase gene was enriched, from 13- and 103-fold to 77- and 802-fold in different reactors with different oxytetracycline concentrations. This result is mainly associated with the functional microbes—*Eubacterium* spp.—belonging to gram-positive bacteria in this system. It has been reported that transposons are important determinants of antibiotic resistance, especially in gram-positive bacteria, on the contrary, integrons appear more frequently in gram-negative bacteria (JR Scott and Churchward, 1995). In addition, transposons commonly carry a tetracycline resistance gene encoding an efflux pump for tetracycline, and transposons' abundance levels are positively associated with oxytetracycline concentration (Zhu et al., 2013). As for integrons, they most commonly contain resistance cassettes carrying the *sul2* gene (Singh et al., 2005), which appeared to be depleted in this study. The above result also confirms that the emergence of ARGs is associated with transposases and integrons (Binh et al., 2008; Zhang et al., 2011). Given that *Tp614* was enriched in both MFCs and microbial controls, it represents a common challenge for different biological processes for wastewater treatment.

4. Conclusion

This study showed the feasibility of using MFCs to enhance degradation of oxytetracycline and eliminate its antibacterial activity. Continuous electrical stimulation and higher ATP concentration mainly contributed to bioelectrochemical reactions more rapidly to enhance oxytetracycline removal in MFCs. The number of ARGs and the normalized copy number of ARGs in biofilms of MFCs were both lower than those in microbial reactors representing conventional anaerobic treatment. Hence, such a process may have its advantage over traditional wastewater treatment process on the minimization of ARGs horizontal transfer and the evolution of novel ARGs through enriching more quickly *Eubacterium* spp. as

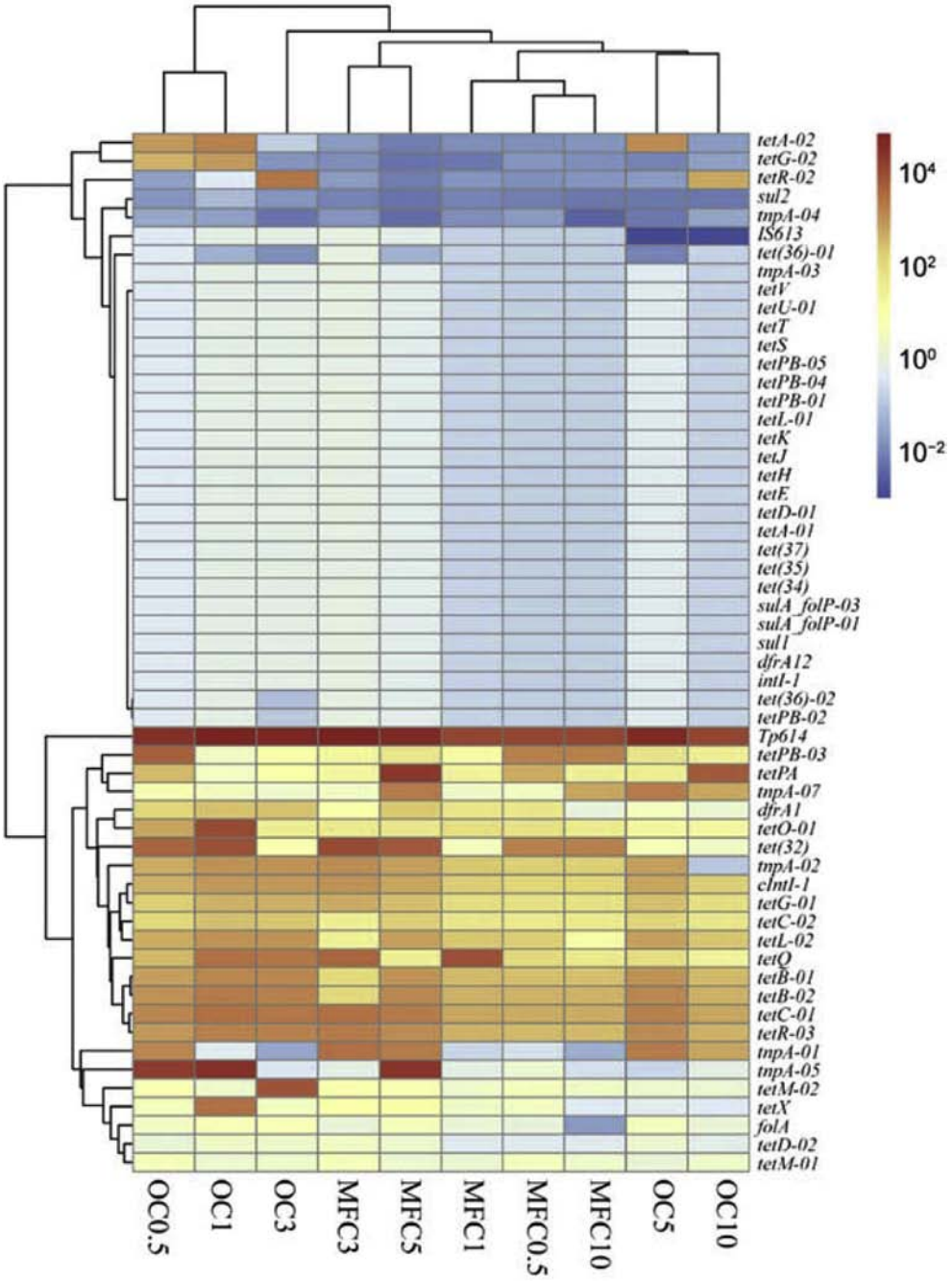

Fig. 5. The enrichment heatmap of ARGs in effluents from the reactors designed for oxytetracycline removal. OC0.5, OC1, OC3, OC5, and OC10 represent effluents from microbial controls containing oxytetracycline at 0.5, 1, 3, 5, and 10 mg/L, respectively; MFC0.5, MFC1, MFC3, MFC5, and MFC10 denote effluent samples from MFCs processing oxytetracycline at 0.5, 1, 3, 5, and 10 mg/L, respectively. The legend shows the fold changes of ARG abundance in effluent samples compared to the sample from raw pig mature. Columns and rows were clustered based on the Bray-Curtis distance.

sole functional bacteria. Nevertheless, *Tp614* as a transposase gene was enriched in both MFCs and microbial reactors, reveling that it may be a common challenge for biological treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.05.047.

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