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

Weifu Yan [a, c], Yunyan Guo [b, c], Yong Xiao [a, **], Shuhua Wang [a, c], Rui Ding [a, c], Jiaqi Jiang [e], Haiyin Gang [a, d], Han Wang [e], Jun Yang [b], Feng Zhao [a, *]

[a] CAS Key Laboratory of Urban Pollutant Conversion, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, 361021, PR China
[b] Aquatic EcoHealth Group, Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 361021, Xiamen, PR China
[c] University of Chinese Academy of Sciences, Beijing, 100049, PR China
[d] College of Environmental Science and Engineering, Hunan University, Changsha, 410082, PR China
[e] College of Ecology and Resource Engineering, Wuyi University, Wuyishan, 354300, PR China

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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-

* Corresponding author.
** Corresponding author.
*E-mail addresses:* yxiao@iue.ac.cn (Y. Xiao), fzhao@iue.ac.cn (F. Zhao).

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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.