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Original Research

Bacterial Communities in a Full-scale Combined A/O+BIOFOR System Treating Pharmaceutical Wastewater

Erming Ouyang[1], Yao Lu[1], Jiating Ouyang[1], Xiaojia Liu[2], Xiaohui Wang[2*]

[1]School of Civil Engineering and Architecture, Nanchang University, Nanchang 330031, China
[2]College of Chemical Engineering, Beijing University of Chemical Technology, Beijing 100029, China

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Abstract

Mixed pharmaceutical wastewater contains high levels of chemical oxygen demand (COD) and high concentrations of toxic and harmful substances. A conventional wastewater treatment process cannot treat this wastewater sufficiently to meet discharge standards. Therefore, mixed pharmaceutical wastewater treatment is a serious challenge to wastewater management. In this study, a full-scale combined anaerobic/oxic (A/O) and biological filtration oxygenated reactor (BIOFOR®) process was used to treat the mixed pharmaceutical wastewater. The objectives were to evaluate the removal efficiency of the combined A/O+BIOFOR process on mixed pharmaceutical wastewater and to examine the bacterial community structures in process. The results showed that the effluent concentration of COD, NH_4^-N, and SS could meet the Chinese mission standard of water pollutants for the pharmaceutical industry mixing/compounding and formulation category (GB21908-2008). MiSeq sequencing data showed that the two systems (A/O and BIOFOR) harbored different bacterial diversity and communities. In phylum level, candidate-division-TM7 was the most predominant phylum in the A/O system, while Proteo bacteria was the most dominant phylum in the BIOFOR reactor. At the genus level, 126 genera were unique in A/O or BIOFOR reactors. The results of this study provided insights into the bacterial community structure and diversity in pharmaceutical wastewater treatment system and can provide reference for the treatment of mixed pharmaceutical wastewater.

Keywords: pharmaceutical wastewater, A/O+BIOFOR process, miseq sequencing, bacterial community

Introduction

Pharmaceutical wastewater is one of the most important sources of industrial wastewater, and it contains a large number of hazardous and toxic substances and has strong bacterial toxicity. Pharmaceutical wastewaters from the manufacturing of antibiotics could significantly impact the environment, as they could interrupt the biological treatment processes in sewage treatment plants because of the recalcitrant and toxic compounds [1-3], leading to the release of incompletely degraded pollutants into the environment.

*e-mail: 379979971@qq.com

The anaerobic/oxic (A/O) process consists of sequential anaerobic and aerobic stages for biological phosphorus removal [4]. The A/O process has been widely used for sewage treatment in both urban and rural areas and is favored for its high efficiency and low energy consumption [5]. The biological filtration oxygenated reactor (BIOFOR) process has high biofilm concentration and strong retention capacity. This process uses a special aeration head that can effectively supply oxygen and is easy to operate and maintain. The distribution of fluid in BIOFOR is completely uniform. The novel combined A/O+BIOFOR process combines the advantages of the two technologies.

Examining the microbial community structures of this novel process is necessary for understanding the complex interactions occurring in the system and finding ways to improve the design and operation of the process [6-7]. Molecular biology techniques have been used to analyze microbial community structures in activated sludge since 1995 [8]. However, because of low throughput, the application of traditional molecular methods was limited [9]. In recent years, with the development of next-generation DNA sequencing techniques, high-throughput sequencing such as Illumina MiSeq sequencing has been introduced to improve the analysis of microbial diversity [10-11].

In this study, a full-scale A/O+ BIOFOR process was used to treat mixed pharmaceutical wastewater. The objectives were to assess the treatment effect of the combined A/O+ BIOFOR process on mixed pharmaceutical wastewater and to examine the bacterial community structures in the aeration tank of A/O process and BIOFOR reactor.

Materials and Methods

Raw Water

The mixed pharmaceutical wastewater was obtained from a medical company located in Jiangxi, China (115.532487°E, 28.048071°N). This company is mainly engaged in the development and production of various types of health care products and external disinfection products. The wastewater is mainly divided into two parts: production wastewater and domestic sewage in the factory. The production wastewater is mainly obtained from the steps of extracting, washing drugs, washing bottles, washing equipment, and washing workshop grounds. The domestic sewage mainly is generated by the production staff, office staff, and the canteen.

Sample Collection

In summer 2015 we collected activated sludge samples from the aeration pool of the A/O process and the BIOFOR reactor once a day for three consecutive days to generate triplicates from a currently functioning sewage treatment facility. In total, six samples were collected, and each sample was dispensed into a 1.5 mL sterile Eppendorf tube and centrifuged at 14,000×g for 10 min. The supernatant was decanted, and the pellets were stored at -20ºC prior to analysis.

DNA Extraction and PCR Amplification

The pellets of the samples were washed three times by centrifuge using sterile high-purify water for 5 min at 15,000 g. DNA extraction was then performed using a Fast DNA SPIN Kit for Soil Kit (MP Biotechnology, USA) according to the manufacturer's protocol. The V4-V5 region of the bacterial 16S ribosomal RNA genes were amplified by PCR (95ºC for 2 min, followed by 25 cycles at 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s, and a final extension at 72°C for 5 min) using primers 515F 5'-barcode-GTGCCAGCMGCCGCGG)-3' and 907R 5'-CCGTCAATTCMTTTRAGTTT-3', where the barcode is an eight-base sequence unique to each sample [12]. PCR reactions were performed in triplicate 20 μL reaction volumes 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 10ng of template DNA.

Illumina MiSeq Sequencing

Amplicons were extracted from 2% (w/v) agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, Madison, WI, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols.

Processing Sequencing Data

Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 300-bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (ii) exact barcode matching, two nucleotide mismatch in primer matching and reads containing ambiguous characters were removed; and (iii) only sequences with 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 (version 7.1 drive5.com/uparse) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (rdp.cme.msu.edu) against the silva (SSU115)16S rRNA database using a confidence threshold of 70% [13].

Statistical Analysis

The Shannon-Wiener index was used to assess bacterial diversity. Detrended correspondence analysis (DCA) was performed to examine the variation among bacterial communities of these six samples. These statistical analyses were performed using the VEGAN package in R (v.2.15.1, r-project.org).

Results and Discussion

Efficiencies of the Combined A/O+BIOFOR Process on Pollutant Removal

During the 181-day study period, the influent COD concentration ranged from 1,618-2,286 mg/L (Fig. 1). The effluent COD concentration remained relatively stable with an average of 52 mg/L (Fig. 1). In general, COD removal rate did not change significantly and saw minor fluctuations around 97.5%. The influent NH_4^+-N concentration fluctuated from 32 to 68 mg/L during the 181-day study period (Fig. 2). The effluent NH_4^+-N concentration showed an increasing trend after 16 days of operation, reaching a maximum of 9.7 mg/L. After day 16, the effluent NH_4^+-N concentration was generally stable. The NH_4^+—Naverage removal rate was 84.51% (Fig. 2). During the first 100 days, the influent suspended solids (SS) concentration fluctuated from 36 to 88 mg/L, with the lowest concentration of 36 mg/L on day 73 (Fig. 3). After day 100, the SS concentrations were relatively stable, ranging from 68 to 85 mg/L. The effluent SS concentration fluctuated from 13 to 28 mg/L and the average SS removal rate was 69.07% (Fig. 3). All these results showed that the combined A/O+BIOFOR were effective in treating the mixed pharmaceutical wastewater; and the three indexes of the effluent met the Chinese discharge standard of water pollutants for the pharmaceutical industry mixing/compounding and formulation category in China (GB21908-2008).

Bacterial Community Structures in the Combined A/O+BIOFOR Process

There were 21,064-28,205 effective reads for the six activated sludge samples (Table 1). The average number of OTUs from A/O system was 1,129, significantly lower than the BIOFOR reactor (1,350) (p<0.05). Similarly, the average Shannon-Wiener index in A/O(5.02) was significantly lower than that in the BIOFOR reactor (5.83). These Shannon-Wiener values are typical for diverse microbial populations without a few strongly dominant tax[14].

Candidate-division_TM7 was the most predominant phyla in the A/O system, accounting for 31.72-33.81% of the total effective bacterial sequences, followed by *Proteobacteria* (19.46-23.76%). However, in the BIOFOR reactor, the most dominant phylum was *Proteobacteria*, accounting for 36.35-46.68% of total effective bacterial sequences (Fig. 4). Similar results were obtained in previous studies, which found that *Proteobacteria* were the dominant members of wastewater microbial communities [15-19]. Previous studies also suggested that the most diverse group of bacteria in 6- and 12-day-old biofilms was *Proteobacteria* [20-21]. The Gram-negative *Proteobacteria* are a major group of bacteria

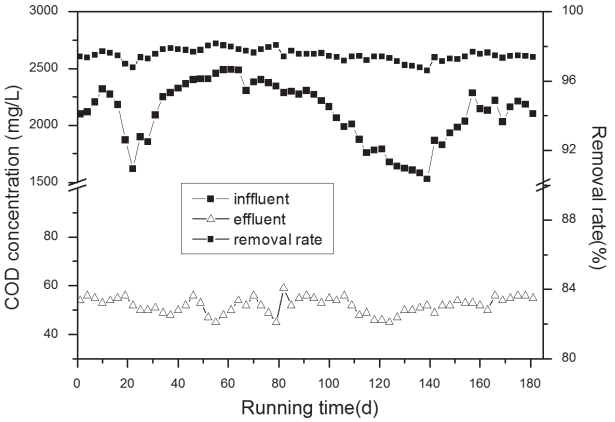

Fig. 1. COD removal by the combined A/O+BIOFOR process.

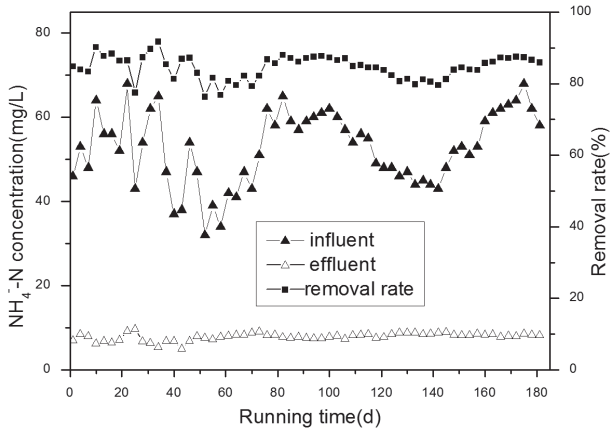

Fig. 2. NH_4^+-N removal by the combined A/O+BIOFOR process.

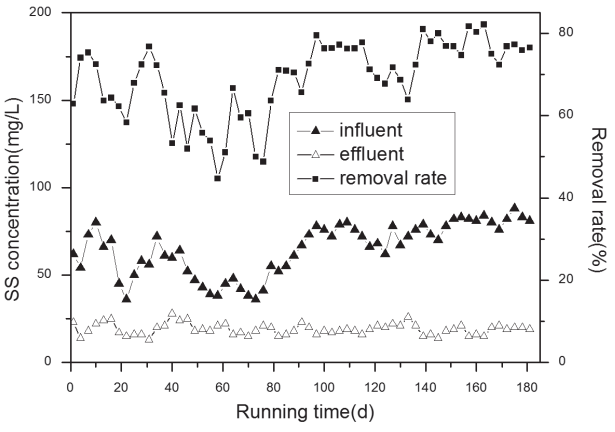

Fig. 3. SS removal by the combined A/O+BIOFOR BIOFOR process.

Table 1. Diversity indices from six samples.

Samples	Sequences	OTUs	Shannon-Wiener
O1	21,064	1,073	4.96
O2	28,179	1,148	5.04
O3	27,350	1,167	5.07
B1	27,876	1,407	6.25
B2	27,257	1,254	5.96
B3	28,205	1,390	6.28

encompassing a wide variety of aerobic, anaerobic, and facultative bacteria [22] that are able to degrade a broad spectrum of organic contaminants and enable biological nitrogen and phosphorus removal [23]. In the BIOFOR reactor, *Firmicutes* was also is the dominant phyla in the BIOFOR reactor (Fig. 4). This observation was consistent with Shi et al. [24], who reported that *Firmicutes* were the predominant group in the anaerobic process for pharmaceutical wastewater treatment. *Chloroflexi* and *Acidobacteria* were highly enriched in both reactors. In addition, the percentages of *Actinobacteria* and *Bacteroidetes*were were similar in the two systems (Fig. 4).

At the genus level, a total of 327 genera were acquired from all six samples. Among the 327 assigned genera, 201 were shared by both A/O and BIOFOR reactors, such as *Ferruginibacter* and *Zoogloea*, and accounted for 71-89% of the classified sequences. Members of *Ferruginibacter*, such as *Ferruginibacter alkalilentus* and *Ferruginibacter lapsinanis*, are capable of hydrolyzing some organic matter. Members of *Zoogloea*, such as *Zoogloea ramigera*, have long been considered the typical activated sludge bacteria responsible for the formation of activated sludge flocculation and to improve the purification process [25]. One-hundred and twenty-six genera were observed only in A/O or BIOFOR reactors, and they accounted for 11-29% of total classified sequences in each sample. Although the two reactor share the majority of the genera, they also harbor some unique genera.

The 15 most abundant genera in each sample were selected (a total of 33 genera for all six samples), and their abundances were compared to those in other samples (Fig. 5). In the A/O system, *Candidate_division_TM7_norank* was the most dominant, and *Anaerolineaceae_uncultured*, *Nitrosomonadaceae_uncultured*, *Caldilineaceae_uncultured*, and *WCHB1-60_norank* were also highly enriched. In the BIOFOR reactors, *Pseudomonas* had relatively high proportions, which have been identified as potential pathogens in a drinking water treatment membrane filtration system [26]. *Geobacter* was found to be dominant in BIOFOR samples, but it was found in limited amounts in A/O samples. Similarly,

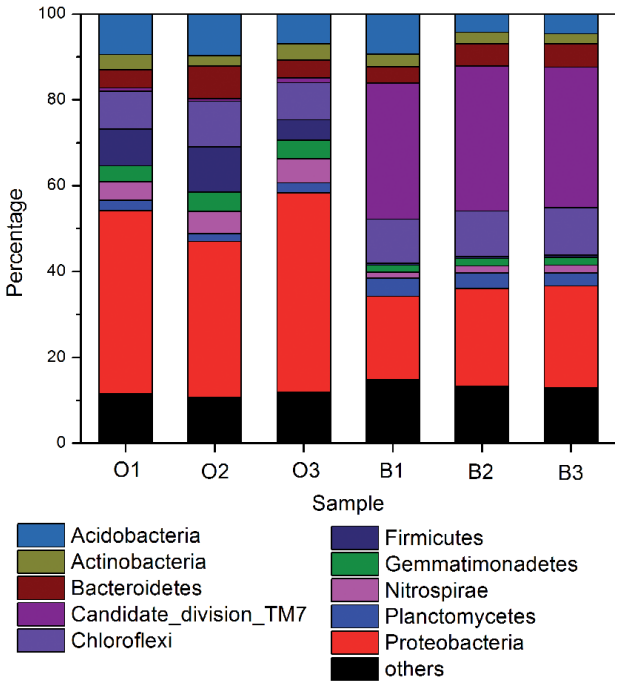

Fig. 4. Abundances of different phyla in the six activated sludge samples. The abundances are presented in terms of percentage of total effective bacterial sequences in a sample. O1, O2, and O3 indicate the three replicate samples from the aeration tank of the A/O system, and B1, B2, and B3 indicate the three replicate samples from the BIOFOR reactor.

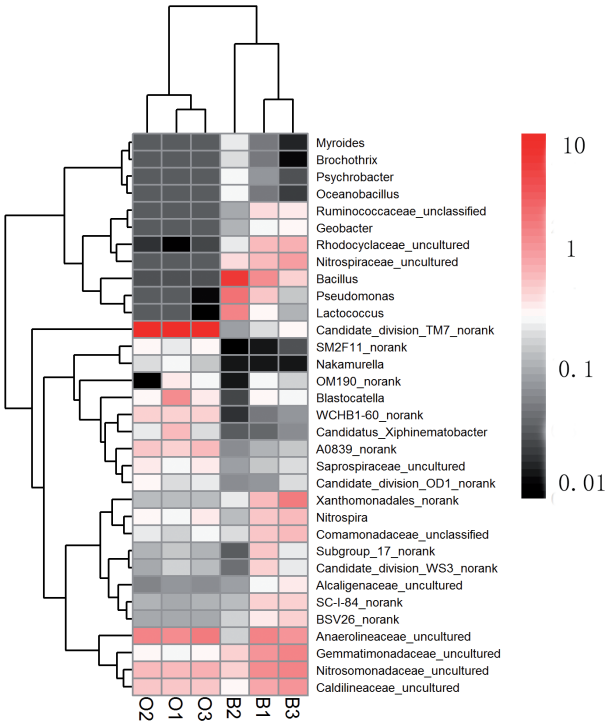

Fig. 5. Heat map of top 15 genera in each sample. Thirty-three genera were selected from six samples; the color intensity in each panel shows the percentage of that genus in the sample in reference to the color key on the right-hand side. O1, O2, and O3 indicate the three replicate samples from the aeration tank of the A/O system, and B1, B2, and B3 indicate the three replicate samples from the BIOFOR reactor.

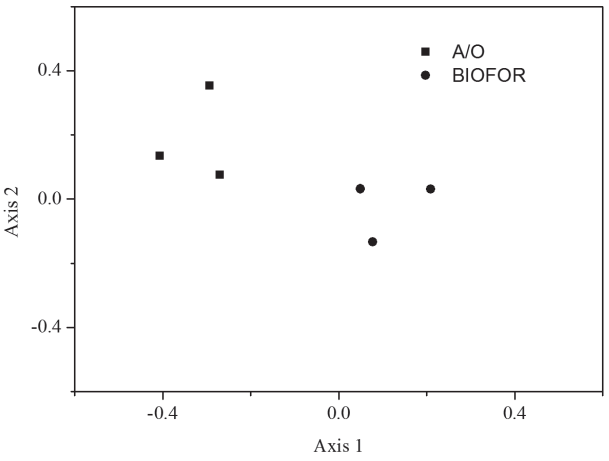

Fig. 6. DCA of bacterial communities from A/O and BIOFOR systems.

the percentage of *Bacillus* in the BIOFOR reactor was higher than that in the A/O system. While the percentage of *Anaerolineaceae_uncultured* in the BIOFOR reactor was lower than in A/O samples. The proportions of *Anaerolineaceae*_uncultured, *Nitrosomonadaceae*_ uncultured, and *Cakdilineaceae* _uncultured in the two reactors were not significantly different.

All these results suggested that the two systems harbor different bacterial communities. The DCA results also showed that the samples from A/O and BIOFOR were distributed in different parts of the data space (Fig. 6).

Conclusions

The A/O+BIOFOR combined processes were suitable for the treatment of mixed pharmaceutical wastewater. The effluent water met the Chinese mission standard of water pollutants for the pharmaceutical industry mixing/compounding and formulation category (GB21908-2008). MiSeq sequencing data showed that the two systems (A/O and BIOFOR) harbored different bacterial communities and diversity. The result also revealed that dominated bacterial communities make great contributions to pollutant removal. The results of this study provided insights into the bacterial community structure and diversity in a pharmaceutical wastewater treatment system and can provide reference for the treatment of mixed pharmaceutical wastewater.

Acknowledgements

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