Changes of Microbial Community and Reactor Performance in Sequencing Batch Reactors Under Diclofenac Selective Pressure

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Abstract: The wide use of diclofenac (DCF) ineluctably increases the release into wastewater that might cause potential negative effects on wastewater treatment system. To investigate whether DCF caused adverse impacts on the wastewater treatment efficiency and changes of microbial community structure, the exposure experiments at three levels (0, 5, and 50 μg L^{-1}) were conducted in sequencing batch reactors (SBRs) for 120 days. Results indicated that 50 μg L^{-1} DCF decreased the chemical oxygen demand (COD) removal by about 10%, but had no obvious effect on the ammonia and total nitrogen removal ($p > 0.05$). 5 μg L^{-1} DCF could improve superoxide dismutase (SOD) and succinate dehydrogenase (SDH) activity, while 50 μg L^{-1} DCF would inhibit SOD and SDH activity. The extracellular polymeric substance (EPS) content increased with the increase of DCF concentration. Compared to the control, Gram-negative bacteria increased and Gram-positive bacteria decreased under 50 μg L^{-1} DCF pressure. Shannon-Wiener index is calculated by PLFA compositions and 16S rRNA gene pyrosequencing indicating that microbial diversity increased in 5 μg L^{-1} DCF but decreased in 50 μg L^{-1} DCF. 16S rRNA gene pyrosequencing results showed that Chloroflexi, OD1, and Firmicutes kept the decreasing tendency with the increase of DCF concentration.

Keywords: Diclofenac, Sequencing batch reactors, Microbial community structure, Microbial iversity, Wastewater treatment efficiency.

1. INTRODUCTION

Diclofenac (DCF), one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs), has a wide range of uses in both human and veterinary medicine. It is also classified as an emerging contaminant often detected in the environment (Praveena *et al*., 2018; Sathishkumar *et al*., 2020). Many previous studies reported that wastewater treatment plants (WWTPs) were the occurrences hotspot of pharmaceuticals and the main resource to their receiving river (Kapelewska *et al*., 2018; Sathishkumar *et al*., 2020). In accordance with their observations, the presence of DCF in the WWTPs has a mean concentration ranging from 0.1 to 3.2 μg L^{-1}.

In the last few decades, the researches focused on the occurrence, removal, and fate of pharmaceuticals in the wastewater treatment process and the biodegradation of pharmaceuticals from wastewater (Aracagök *et al*., 2018; Kapelewska *et al*., 2018; Sathishkumar *et al*., 2020; Fernandez-Fontaina *et al*. 2016). Sui *et al*. (2011) studied the occurrence of 12 pharmaceuticals in two WWTPs using three biological treatment processes in Beijing and results indicated that different processes had significant effects on the removals of pharmaceuticals. The median removal of DCF was 61% by MBR, 37% by CAS and 21% by BNR. DCF was poorly removed (less than 20%) in a full-scale anaerobic/anoxic/aerobic process combined with membrane bioreactor for municipal wastewater (Xue *et al*. 2010). Due to the high frequency detection, low removal efficiency and high ecological risk, DCF the European Union (EU) included it in the watch list of substances that requires environmental monitoring in EU member states (Vieno and Sillanpaa 2014).

Most municipal WWTPs use biological treatment which is the microbial component of the activated sludge treatment process to remove nitrogen and organic pollutants. Low removal efficiency in WWTPs results in DCF still existing in the wastewater, which may bring more potential impact on organisms in the treatment processes. Thus, it is important to determine if the presence of pharmaceuticals in wastewater has a negative influence on activated sludge in microbial communities thereby impacting treatment performance. Many studies were conducted on the impacts of different conditions, such as hydraulic retention time, sludge retention time, nitrification, denitrification and heterotrophic conditions, on the pharmaceutical biotransformation, removal and biodegradation (Fernandez-Fontaina *et al*. 2016, Kruglova *et al*. 2014, Fernandez-Fontaina *et al*. 2012, Suarez *et al*. 2010). Kruglova *et al*. (2014) reported that the biodegradation

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of DCF appeared a high fluctuation, which might be caused by the development of nitrifying activated sludge. It has been demonstrated that the increase of nitrifiers could enhance the biodegradation of DCF (Fernandez-Fontaina *et al*. 2012). Kraigher *et al*. (2008) indicated that diversity indices of bacterial community reduced in the reactor supplied with 50 μg L^{-1} pharmaceuticals, which studied the influence of pharmaceutical residues at the concentrations of 0, 5, 50, 200, and 500 μg L^{-1} on bacterial communities by Terminal restriction fragment length polymorphism (T-RFLP). However, there is little information on the relationship among reactor performance, extracellular polymeric substance (EPS) of activated sludge and microbial community.

In this study, the laboratory-scale sequence batch reactors (SBRs) were operated for 120 d to culture the activated sludge under DCF selective pressure. During the operation period, the contents of chemical oxygen demand (COD), ammonia (NH_4^+-N), total nitrogen (TN) and EPS were measured regularly. The enzymes activity and microbial community in activated sludge were analyzed after achieving steady state as well. The objectives of this study were (1) to determine the changes of microbial activity and community based on key enzyme activities and high-throughput 16S rRNA gene sequencing; (2) to understand the effects of effluent quality with DCF presence; and (3) to learn more information about the influences of nitrification under the pressure of DCF. This study contributes new information concerning the DCF effect on microbial community composition and its relationship to the removal of other contaminants in the wastewater treatment process.

2. MATERIALS AND METHODS

2.1. Reagents

Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade methanol, HPLC-grade acetonitrile and HPLC-grade acetic acid were supplied by Merck (Darmstadt, Germany). Milli-Q water, with a resistivity of at least 18.2MΩ·cm, was produced from a Millipore purification system (Billerica, CA, USA).

2.2. Bioreactor

Exposure experiments were conducted in SBRs that had a working volume of 4 L. The reactors were inoculated with activated sludge collected from the secondary sedimentation tank in a municipal WWTP in Jiangsu, China. The SBRs operated at 21 ± 1 °C with three cycles each day. Each cycle (8 h) consisted of a 60 min feeding period (the synthetic wastewater was pumped into the reactor), 330 min of aeration, 30 min of settling, 10 min of decanting and a 50 min idle period. The amount of effluent discharged from each SBR in each cycle was 2.4 L. The quantified DCF stock suspension was added to two reactors during the feeding period with the initial DCF concentration controlled at 5 μg L^{-1} (R_5) and 50 μg L^{-1} (R_{50}). A reactor operating without DCF served as a control (R_0). The complete mixture of synthetic wastewater is shown in Table **1**. During the whole study, the initial pH was controlled at 7.0 - 8.0. The sludge retention time (SRT) was controlled at 20 days by adjusting the sludge discharge circle, a hydraulic retention time was about 13.3 h, and the mixed liquor suspended solid (MLSS) concentration was 3.5 ± 0.2 g L^{-1}. The initial concentrations of chemical oxygen demand (COD), NH_4^+-N and PO_4^{3-}-P were 300, 20, and 3 mg L^{-1},

Table 1: Composition of Synthetic Wastewater (Modified from (Kruglova *et al*., 2014))

Substance	Concentration (mg L^{-1})	Composition of Nutrient Solution	Concentration (mg L^{-1})
CH_3COONa	90	$FeCl_2·6H_2O$	1500
NH_4Cl	35	H_3BO_3	150
KH_2PO_4	30	$CuSO_4·5H_2O$	30
$CaCl_2·2H_2O$	60	KI	180
$MgSO_4·7H_2O$	200	$MnCl_2·4H_2O$	120
$NaHCO_3$	218	$(NH_4)_6MoO_{24}·4H_2O$	40
Nutrient solution	0.3 mL	$ZnSO_4·7H_2O$	120
Diclofenac	10	$CoCl_2·6H_2O$	150
		EDTA	10000

respectively, which accorded to a municipal wastewater composition.

During the operational period, samples of influent and effluent were taken on a regular basis. Wastewater samples were filtered through syringe nylon membrane filters (0.45 μm pore-size) to remove biomass. The concentration of COD, NH_4^+, NO_3^-, NO_2^-, total nitrogen (TN) and MLSS were determined by standard methods (Federation and Association 2005). DO concentration, pH, and temperature values were measured by oxygen (SG6, METTLER TOLEDO Inc., USA) and pH meters (FE20, METTLER TOLEDO Inc., USA).

2.3. Determination of Key Oxidation Enzyme Activities and EPS in Activated Sludge

The activities of superoxide dismutase (SOD) and succinate dehydrogenase (SDH) extracted from activated sludge were detected. The SOD and SDH activity were evaluated by corresponding detection kits pursued from Nanjing Bioengineering Institute (China). All enzyme activities were normalized back to total protein concentration. The protein was measured by a Micro BCA Protein Assay Kit pursued from Nanjing Bioengineering Institute (China).

EPS are composed of a mixture of macromolecules, such as protein, polysaccharides, humic-like substance and nucleic acids (Ding *et al*. 2015). Generally, the main contents of EPS are represented by exopolysaccharide (PS) and their protein (PN) content (Zhu *et al*. 2015). In this study, EPS in activated sludge was extracted using the thermal treatment method (Wang *et al*. 2009). PS content (glucose equivalent) was determined using the phenol-sulfuric method with glucose as the standard (Dubois *et al*. 1956). PN content was measured by a modified Lowry method using bovine serum albumin as a standard (Frolund *et al*. 1996).

2.4. Phospholipid Fatty Acid (PLFA) Extraction and Statistical Analysis

The PLFA extraction method was described by (Niu *et al*. 2012). After extraction, the resulting fatty acid methyl esters were prepared according to MIDI protocol and detected by Agilent 7890 GC (USA). The results were analyzed using the MIDI Sherlock Microbial Identification System (MIDI, Newark, DE) (Xue *et al*. 2008).

The diversity of fatty acid was indicated by the Shannon-Wiener index (Niu *et al*., 2012), which is generally defined as:

$$H = -\sum_{i=1}^{s} p_i \ln(p_i) \quad (1)$$

where H is Shannon-Wiener index, s is the total number of PLFA in each sample and is the percentage of the peak area of PLFA to the total area of each sample. In this research, the GC assay peak areas were denoted to calculate values of p_i for each sample, which was inserted into equation (1).

2.5. Procedure of 16S rRNA Gene Amplicon Sequencing

Activated sludge collected from the bottom of three reactors (R_0, R_5 and R_{50}) made up the 16S rRNA gene amplicon sequencing sample. Processes were described as follows: DNA extraction, 16S rRNA gene PCR amplification and PCR products purification. To amplify and sequence the V1V2 hypervariable region of the 16S rRNA gene, forward primer (50-AGAGTTTGATYMTGGCTCAG-30) and reverse primer (50-TGCTGCCTCCCGTAGGAGT-30) were selected and different 8-base barcodes and a Guanine were linked to the 50 end of each primer (Zhu *et al*., 2015). Then the purified products were sent for sequencing using Illumina high throughput sequencing platform (Miseq, Illumina Inc., USA).

The acquired sequencing data were processed by the Sickle and Mothur program to remove the low quality of sequence and reduce noises. Lastly, the filtered sequences were assigned a taxon by the RDP classifier.

2.6. Data Analysis

Averages and standard errors were determined for all value results. One-way analysis of variance (ANOVA) was used to assess the homogeneity of variance with a significance level of 5% ($p < 0.05$). All statistical analyses were performed using SPSS statistics 22.0.

3. RESULTS AND DISCUSSION

3.1. Reactor Performance

The initial concentration of COD and NH_4^+-N for the reactor were maintained at 300 and 20 mg L^{-1}, respectively. Schnell *et al*. (2000) reported that the stable stage of reactor operation usually was attained after at least two SRT of adaption in the individual biological processes. In this study, the stable stage was achieved after the three SRT (60^{th} d). COD, NH_4^+-N and TN removal of the stable stage were presented in Table **2**. As shown in Table **2**, COD removals in R_0-R_{50}

Table 2: Removals of COD, NH_4^+-N and TN in Reactors at the Stable Stage

Reactor Code	COD Removal (%)	NH_4^+-N Removal (%)	TN Removal (%)
R_0	88.27±3.17	99.39±0.35	70.14±7.27
R_5	81.45±3.29*	99.31±0.30	70.30±5.74
R_{50}	77.64±2.20*	99.30±0.34	67.10±3.74

*indicating a statistically significant difference compared to R_0 ($p < 0.05$).

at the stable stage were 88.27±3.17%, 81.45±3.29% and 77.64±2.20%, respectively. NH_4^+-N removals were efficient over 99% and TN removal were from 67.10% to 70.30% in the reactors. The removals of NH_4^+-N and TN had no differences in all reactors. Results indicated that environmental concentration DCF affected the overall COD removal. Similarly, Grekova-Vasileva and Topalova (2014) also reported a decreasing COD removal (51%), which was registered during the shock loading of the activated sludge with 0.033 mg mL^{-1} azo-compounds. The high nitrification performance might be caused by the COD content. Under the pressure of DCF, NH_4^+-N and TN removal had no significant differences ($p > 0.05$). The results might be caused by the low concentration of DCF. Amorim *et al*. (2014) found that nitrogen removal was not affected by the presence of ofloxacin, norfloxacin and ciprofloxacin at concentrations of 9 and 32 uM. The effluent water quality under the long-term presence of environmental concentration DCF needs to be further studied.

Table **3** shows the average DCF removal in the stable stage of each sample. As shown in Table **3**, the DCF removals were 57.64±2.92% and 59.45±0.44% in R_5 and R_{50}, respectively. The results of DCF removal are similar to the average level reported by other studies (Duan *et al*. 2013), which observed that DCF was moderately removed by 37-53% in a Shanghai WWTPs with the anaerobic/anoxic/oxic activated sludge process. Xue *et al*. (2010) reported that DCF removal rate generally depended on sludge conditions and positively related to the DO level and DCF was largely removed in anaerobic tank in a WWTP with the full-scale anaerobic/anoxic/aerobic process combined with membrane bioreactor. DCF was hard to adsorb to sludge due to its ionization under neutral experimental conditions (Xue *et al*. 2010). Kruglova *et al*. (2014) also found that DCF was moderately biodegradable ($0.5<k_{biol}<1$) with influent concentration of 1.0±0.4 μg L^{-1}. It seems reasonable to infer that DCF removal mostly rely on biodegradation in this study. The similar DCF removal in R_5 and R_{50} might be caused by a similar microbial community structure.

3.2. Key Oxidation Enzyme Activities in the Activated Sludge

SOD is one of the important antioxidant enzymes and indicators of oxidative stress (Diniz *et al*. 2015). SDH plays an important role in the oxidation process (Sastre 2003). SOD and SDH were extracted from activated sludge at the 100^{th} day to evaluate the oxidation stress of microorganisms. Figure **1** shows the changes of SOD and SDH activity. It is evident from Figure **1** that the activity of SOD (U mg $protein^{-1}$) increased from 32.16±0.14 (R_0) to 34.56±0.09 (R_5) but decreased to 21.6±0.24 (R_{50}). The activity of SDH (U mg $protein^{-1}$) had a similar change trend with SOD, that is, changing from 5.44±0.06 (R_0) to 10.25±0.12 (R_5) and 2.47±0.04 (R_{50}). The results suggested that the 5 μg L^{-1} DCF could improve SOD and SDH activity, while the 50 μg L^{-1} DCF would inhibit SOD and SDH activity. This can be likened to hormesis (a beneficial effect from something normally considered toxic) (Bellavite *et al*. 2010). SOD is the major enzyme that clears the free oxygen radicals and, therefore, reduces the toxicity associated with these radicals (El-Megharbel *et al*. 2015). Zhang *et al*. (2015) illustrated that SOD activity is relatively sensitive to wastewater toxicity. SDH, as a key enzyme in the tricarboxylic acid cycle, is

Table 3: Average DCF Removal in the Stable Stage of each Sample (100 d)

Sample	Influence (μg L^{-1})	Effluent (μg L^{-1})	Average Removal (%)
R_0	--	--	--
R_5	4.78±0.72	2.01±0.16	57.64±2.92
R_{50}	48.96±1.12	19.85±0.24	59.45±0.44

widespread in many prokaryotic cells, which provides electron for multiple prokaryotic cells in the respiratory chain. Bag *et al*. (1999) also found that the SDH activity was inhibited under heavy metals, pesticides, and other organic pollutant pressure, which explains the decreased aerobic respiratory process.

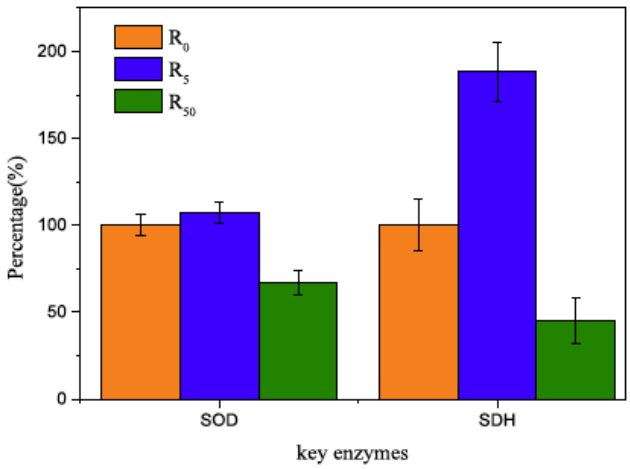

Figure 1: Key oxidation enzyme activities of three reactors in the activated sludge.

3.3. Change of EPS

Generally, the main contents of EPS were represented by exopolysaccharide (PS) and the protein (PN) content (Zhu *et al*., 2015). Figure **2** shows the changes of the sum of PN and PS in activated sludge at the stable stage (100^{th} d). The increasing concentration of PN and PS in three reactors was observed. The concentration of PN and PS were 85.41, 110.54 and 110.44 mg g^{-1} VSS for PN in R_0, R_5 and R_{50}, respectively; 23.81, 29.85 and 30.25 mg g^{-1} VSS for PS in R_0, R_5 and R_{50}, respectively. These results indicate that increasing EPS content was a protective response of microorganisms to the addition of DCF and toxicity of 5 μg L^{-1} DCF may induce the augmentation of EPS content, while the toxicity of DCF causes the reduction. The conclusion was supported by Quan *et al*. (2015), which reported that low toxicity may induce the augmentation of EPS in sludge, but high toxicity may inhibit its production. It was often accepted that the production of EPS was in response to environmental stress (Delgado *et al*. 2010). Wang *et al*. (2016a) reported that bacteria could secrete more EPS content to decrease the heavy metal toxicity to bacteria and the increase of Cd(II) concentration caused the increase of EPS content. Delgado *et al*. (2010) also concluded that microorganisms produce bound EPS as a protective barrier for better survival in the presence of cydophosphamide drugs. The phenomena may be also supported by the changes in oxidation enzyme activities (SOD and SDH), which increased in R_5 but inhibited in R_{50}.

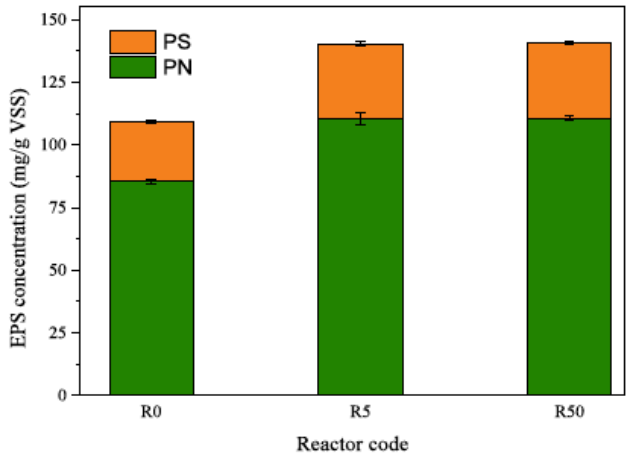

Figure 2: Changes of EPS in samples during the stable stage (100^{th} d).

3.4. Community Diversity by PLFA Analysis

PLFA profiles can be used to analyze the microbial community structure because a relative abundance of characteristic PLFA is different among specific groups of microorganisms (Li *et al*. 2010). The distribution of the carbon chain length of PLFA was mainly between C11 and C20. The dominant PLFA were 16:0, 18:0, 16:1 w7c and 18:1 w7c. Li *et al*. (2010) also reported that 16:0 fatty acids were determined in different filtered media of constructed wetland. In addition, the specific fatty acids, 14:0 anteiso, 16:0 iso 3OH, 15:1 w8c and 18:1 w5c, were only found under 50 μg L^{-1} DCF pressure. These specific fatty acids probably were generated under adverse environmental conditions. Imran *et al*. (2015) examined the microbial community composition of different azo dyes in soil by analyzing PLFA profiles, which also found some specific fatty acids generated under the azo dye pressure.

To know the microbial community diversity by using the PLFA index, the Shannon-Wiener diversity analysis was applied. The Shannon-Wiener diversity indexes are calculated by PLFA compositions described as 2.46 (R_5) > 2.32 (R_0)> 2.29 (R_{50}). The results might be caused by the lower toxicity of low concentration of DCF (5 μg L^{-1}) and the adaptability of microorganisms. Zhang *et al*. (2016) also reported that the diversity of bacterial communities in wetland mesocosms receiving 250 μg L^{-1} ibuprofen-enriched wastewater was significantly decreased (p < 0.05). Some further investigations are needed to study the relationship of

PLFA diversity and DCF concentration, to further define the DCF concentration threshold value.

Generally, PLFA markers represent three microbial groups: Gram-positive bacteria, Gram-negative bacteria and microeukaryotes. Figure **3** describes the changes of predominant microbial groups in three reactors. As shown in Figure **3**, microeukaryotes content had little effect, and Gram-negative bacteria had a greater effect than Gram-positive bacteria, which may be caused by the higher unsaturated fatty acids content of the cell membrane for Gram-negative bacteria (Niu *et al*., 2012). There was little difference in Gram-positive bacteria and Gram-negative bacteria between R_0 and R_5 ($p > 0.05$). Compared to the control (R_0), Gram-negative bacteria increased from 29.03% to 35.7% and Gram-positive bacteria decreased from 11.46% to 5.68% under 50 μg L^{-1} DCF pressure. The results could be explained by the fact that Gram-negative bacteria can adapt to activity sludge disturbance with DCF by changing lipid composition, while Gram-positive bacteria is so stable and rigid that they are unable to adapt to the environmental stress. Moll *et al*. (1999) reported that the monounsaturated fatty acids (Gram-negative bacteria) were more fluid, so Gram-negative bacteria can increase to maintain membrane fluidity as the water temperature decreases. Paje *et al*. (2002) used the isolation techniques to illustrate that 100 μg L^{-1} of DCF can have a selective effect, inhibiting the growth of Gram-positive bacteria.

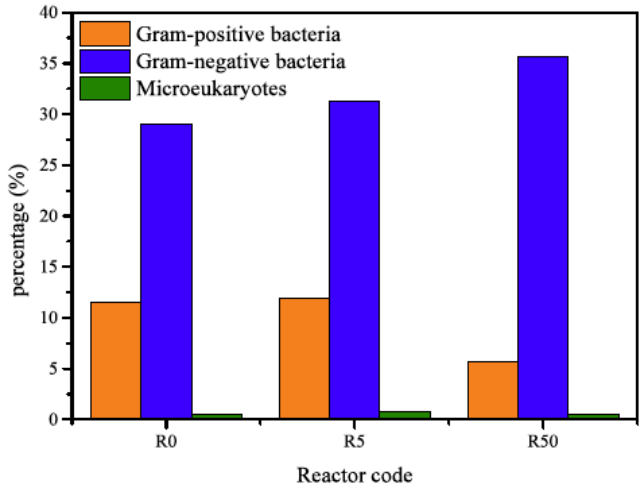

Figure 3: Changes of predominant microbial groups in reactors.

3.5. Changes of the Microbial Community at the Phylum Level

Figure **4a** shows the distribution of dominant bacteria at the phylum level in sludge samples at a stable stage by 16s rRNA gene sequencing analysis. Proteobacteria was determined to be the most abundant phylum in all samples, accounting for 49-81% of the total effective bacterial sequences. This is similar to the analytical results of bacterial communities in activated sludge from 14 sewage treatment plants (Zhang *et al*. 2012), in which Proteobacteria was also the most dominant community. The other dominant phyla were Bacteroidetes and Chloroflexi. These three groups accounted for 63–92% in bacterial communities analyzed in this study. Zhang *et al*. (2016) reported that Proteobacteria could adapt best in response to changes of the external environment in wetland systems. It might be reasonable that Proteobacteria is the most dominant phylum in many situations. Kraigher *et al*. (2008) reported that the role of Chloroflexi in WWTPs was not well understood, but many studies illustrated that Chloroflexi consuming primarily carbohydrates was also one of the dominant phylum (Kraigher *et al*. 2008, Miura *et al*. 2007, Kragelund *et al*. 2007). In this study, the types of phyla are 90% similar in R_0, R_5 and R_{50}, while the abundances of each phylum are significantly different. In the control (R_0), the percentages of Chloroflexi, OD1 and Firmicutes are 10.98%, 4.22% and 1.95%, respectively. With the addition of DCF, Chloroflexi abundances reduced to 4.99% (R_5) and 1.38% (R_{50}). OD1 abundances decreased to 0.72% (R_5) and 0.05% (R_{50}). Firmicutes abundances decreased to 1.36% (R_5) and 0.95% (R_{50}). The variation abundances of different phyla in each sludge samples indicated that the presence of DCF could impact the microbial community in the SBRs. The decrease of COD removal might be caused by the reduction of Chloroflexi, OD1 and Firmicutes. In most constants, degradation of some widely used pharmaceuticals mostly relies on the inherent ability of sludge communities (Kraigher *et al*. 2008). Therefore, the similar DCF removals in R_5 and R_{50} might be caused by the unchanged microbial types at the phylum level. But higher pharmaceutical concentration or longer exposure time with the presence of pharmaceuticals might lead to greater structural shifts, which needs further investigations.

Since proteobacteria were the most dominant bacteria in the phylum level in each sludge sample, a more detailed study about proteobacteria at the class level has been needed for a long time. Figure **4b** described the distribution of proteobacteria in each sludge sample at the class level. Within Proteobacteria, Figure **4b** shows that β-proteobacteria (25.9-47.7%) was the most dominant class in all samples, followed by γ-proteobacteri, α-proteobacteria and δ-

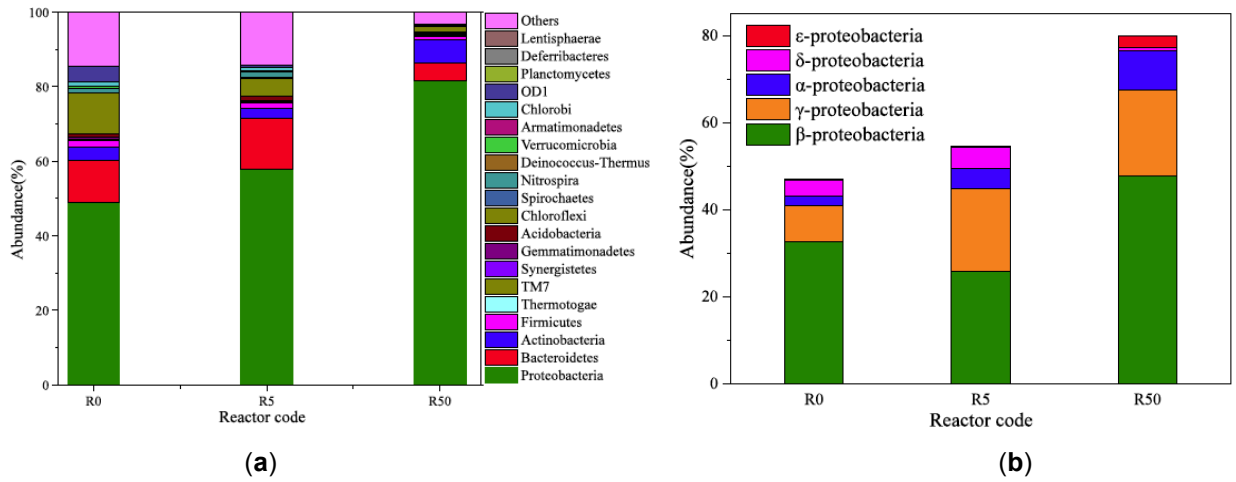

Figure 4: Distribution of dominant bacteria in phylum level (**a**) and five kinds of proteobacteriain (**b**) in sludge samples.

proteobacteria. The abundance of ε-proteobacteria only occurred at very low levels and the maximum value was in R_{50}. Most of them increased under DCF selective pressure except for δ-proteobacteria. The results suggest that the microbial community structure was influenced by the addition of DCF and the detailed changes of microbial community related to the concentration of DCF (Kraigher *et al*. 2008, Lawrence *et al*. 2007). In addition, many previous researchers reported that β-proteobacteria was the dominant class (Zhu *et al*., 2015; Kraigher *et al*., 2008; Lawrence *et al*., 2007). Lawrence *et al*. (2007) concluded that DCF had potential as a selective agent, altering microbial community composition and also found that γ-proteobacteria significantly increased when DCF (10 and 100 μg L^{-1}) was added into the reactor. During the biological treatment processes, some microorganisms could adjust to the changing environment, while others could not adapt and gradually reduced.

3.6. Changes of the Microbial Community at the Genus Level

The heat map was applied to identify the bacterial community structures at the genus level (Figure **5**). As shown in Figure **5**, some genus only detected by the addition of 50 μg L^{-1} DCF, that is, *Nakamurella*, *Micropruina*, *Caloramator, Caldilinea*, *Bdellovibrio*, *Arthrobacter* and *Microbacterium*. Among them, *Micropruina* and *Arthrobacter* belong to *Acidobacterium*, which were probably stimulated by acid pharmaceuticals (such as DCF). Under DCF pressure, *Thauera* decreased from 3.1% to 0.97%, which has been reported as representing denitrifying bacteria in wastewater treatment processes (Tarlera and Denner, 2003), and Suarez *et al*. (2010) reported that removal of DCF was positively affected by the development of nitrification biomass. *Nitrospira* were 1.6% and 0.24% for R_5 and R_{50}, respectively, which suggested that *Nitrospira* was inhibited by 50 μg L^{-1} DCF pressure. Dokianakis *et al*. (2004) found that some pharmaceuticals (including DCF and clofibrate) inhibited the nitrite reduction rate performed by nitrite-oxidizing bacterial culture isolated from activated sludge. In accordance with these studies, it seems reasonable to infer that DCF could inhibit the microorganisms of nitrification and denitrification. In addition, five genera were abundant (> 1%) in at least two samples, including two genus extensively reported in activated sludge, that is, *Dechloromonas* (3.7-35.5%, averaging at 13.8%) and *Zoogloea* (4-13%, averaging at 7.6%), plus three not well-described genera, that is, *Haliscomenobacter* (0.25-3.4%, averaging at 1.9%), *Thauera* (0.97-3.1%, averaging at 1.96%) and *Propionivibrio* (0.8-1.8%, averaging at 1.2%). *Dechloromonas* increased from 3.8% (R_0) to 35.5% (R_{50}) and is a genus capable of reducing perchlorate (Laurie *et al*., 2001). It is frequently reported to assist phosphate accumulating organisms in enhanced biological phosphorus removal reactors (Liu *et al*., 2005). *Zoogloea* decreased from 13.1% (R_0) to 4.2% (R_{50}), which could form characteristic cell aggregates embedded in extracellular gelatinous matrices and have the ability to block toxic compounds using their exocellular matrix. This indicates that the block toxic compounds of the activated sludge were inhibited by 50 μg L^{-1} DCF pressure.

3.7. Changes of Microbial Diversity

Shannon-Wiener index was used to evaluate the microbial diversity of 16S rRNA gene pyrosequencing. The indexes were 1.65 (R_0), 1.75 (R_5) and 1.60 (R_{50}), respectively. The diversity increased with the addition

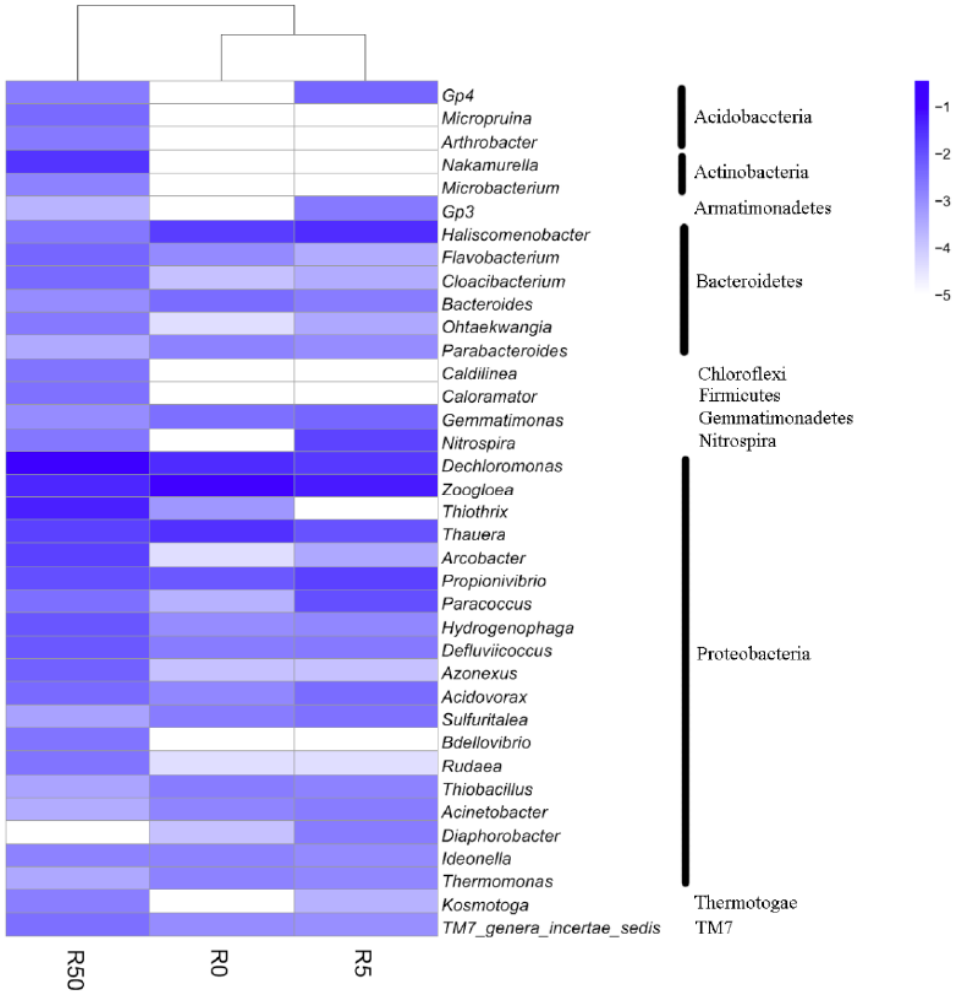

Figure 5: Distribution of dominant bacteria with relative abundance of more than 0.2% according to genus levels in sludge samples.

of 5 μg L^{-1}, while decreased to the presence of 50 μg L^{-1} DCF. Similarly, Wang *et al*. (2016b) found that the microbial diversity inhibited by the 50 μg L^{-1} pharmaceuticals in an aerobic granular sludge membrane bioreactor. The result indicated that pharmaceuticals could cause toxicity to microorganisms, which was also supported by the result of EPS content. With the addition of 50 μg L^{-1} pharmaceuticals, reduced diversity of activated sludge microbial community was observed in small-scale wastewater treatment bioreactors compared to the control (Kraigher *et al*. 2008). The result further proved the microbial diversity result analyzed by PLFA profiles.

4. CONCLUSIONS

In the present work, three SBRs were exposed at environmental concentration DCF to investigate their interaction. After 120 d exposure, nutrient removal under DCF pressures has no differences except for COD. The decrease of COD removal may be caused by the reduction of Chloroflexi, OD1 and Firmicutes consuming primarily carbohydrates. The enzyme activity changes are consistent with the EPS content, increasing in R5 but decreasing in R50. 16S rRNA gene pyrosequencing results reveals that 5 or 50 μg L-1 DCF levels could affect the detailed microbial community structure and composition. Compared to the control, Gram-negative bacteria increased and Gram-positive bacteria decreased under 50 μg L-1 DCF pressure. The microbial diversity is stimulated with the addition of 5 μg L-1 DCF but inhibited at the presence of 50 μg L-1 DCF by Shannon-Wiener indexes of PLFA data and 16S rRNA gene pyrosequencing values.

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