Impacts of typical pharmaceuticals and personal care products on the performance and microbial community of a sponge-based moving bed biofilm reactor

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Abstract

Four lab-scale moving bed biofilm reactors (MBBRs) were built to treat simulated wastewater containing typical pharmaceuticals and personal care products (PPCPs). The efficiency in removing different PPCPs at different concentrations (1, 2 and 5 mg/L) and their effects on the performance of MBBRs were investigated. Results showed that the average removal efficiencies of sulfadiazine, ibuprofen and carbamazepine were 61.1±8.8%, 74.9±8.8% and 28.3±7.4%, respectively. Compared to the reactor without PPCPs, the total nitrogen (TN) removal efficiency of the reactors containing sulfadiazine, ibuprofen and carbamazepine declined by 21%, 30% and 42%, respectively. Based on the microbial community analysis,

increasing the PPCPs concentration within a certain range (< 2 mg/L) could stimulate microbial growth and increase microbial diversity yet the diversity reduced when the concentration (5 mg/L) exceeded the tolerance of microorganisms. Furthermore the presence and degradation of different PPCPs resulted in a different kind of microbial community structure in the MBBRs.

Keywords: moving bed biofilm reactor; pharmaceuticals and personal care products; nitrogen removal; microbial diversity; microbial community structure

1. Introduction

With the increasing use of personal care products (PPCPs) throughout the world, the pollution of water environments caused by PPCPs has become a major issue of social concern (Song et al., 2019). PPCPs are characterized by high toxicity, strong bioaccumulation and refractory degradation in the environment, and can be ingested in the human body and aquatic and terrestrial organisms for a long time (Melvin and Leusch, 2016). Even conditions of μg/L and ng/L will have serious effects on human health and that of the ecological environment (Fatehifar et al., 2018). As an important link between the water cycle and water pollution control, wastewater treatment plants (WWTPs) are the important way for PPCPs to be transferred to the environment (Tran et al., 2013; Zhang et al., 2018b). However, the main purpose of the existing sewage treatment process design and operation is to remove organic matter, nitrogen, phosphorus and other conventional pollutants in sewage (Mohapatra et al., 2016), which is currently inefficient for the full removal of PPCPs (Casas et al., 2015). Therefore, effective control of PPCPs with effluent discharge of the WWTPs is the key to controlling the environmental impact of these PPCPs and ensure water is safety to drink.

WWTPs usually adopt a traditional activated sludge treatment process, which cannot

effectively withstand the impact of excessive discharge of PPCPs (Luo et al., 2014). The removal efficiency varied according to the physicochemical properties of compounds as well as environmental conditions, for example the biological reactor configuration and operational parameters, i.e. hydraulic retention time, sludge retention time and pH (Wang and Wang, 2016). So at present, some WWTPs have begun to use the biofilm process or membrane bioreactor to treat PPCPs, in which the moving bed biofilm reactor (MBBR) has been widely recognized as a potential technology for removing pharmaceuticals (Casas et al., 2015; Fala's et al., 2013).

The MBBR is a new and efficient wastewater treatment process which combines the advantages of activated sludge process and biofilm process (Zhang et al., 2017). This MBBR relies on aeration in the tank and lifting the water flow to put the biocarriers in a fluidized state (Casas et al., 2015). Doing so not only provides macro and micro aerobic and anaerobic environments, but also solves the tensions between autotrophic nitrifying bacteria, heterotrophic denitrifying bacteria and heterotrophic bacteria about dissolved oxygen (DO) and carbon source (Abtahi et al., 2018; Song et al., 2018). The MBBR has many characteristics, such as flexible operation, little water loss, impact on load resistance, prolonged mud age, less residual sludge, no need for regular backwashing, etc. (Chu and Wang, 2011). For these reasons it is regarded as a promising sewage treatment technology.

MBBR not only treats domestic sewage using a low C/N, it can also effectively remove these PPCPs. Guo et al. (2019) undertook a laboratory-scale sequencing batch reactor (SBR) and two MBBRs with different types of biocarriers, the objective being to treat primary wastewater effluent with a low C/N ratio. Their results showed that chemical oxygen demand (COD) and NH_4^+-N were effectively removed by the MBBRs but not by the SBR. Derakhshan

et al. (2018) investigated a MBBR reactor on a laboratory-scale for simultaneous removal of atrazine, organic carbon, and nutrients from wastewater. Their results revealed that the maximum removal efficiencies of atrazine, COD, total phosphorus (TP) and TN were 83.57%, 90.36%, 90.74% and 87.93%, respectively.

There are high concentrations of PPCPs in medicine/healthcare-related wastewater, hospital wastewater and agricultural water, and such wastewater is discharged into municipal pipelines without treatment or unacceptable standard treatment. This greatly affects the quality and quantity of municipal domestic sewage. Currently, most scholars have studied the removal of low concentration PPCPs but only a few studies to work on the removal of high concentrations of PPCPs. A membrane bioreactor was used by Dai et al. (2018) to treat ciprofloxacin contaminated artificial wastewater at three different ciprofloxacin dosages (0, 5 and 10 mg/L). Fatehifar et al. (2018) built an 8.5L aerobic MBBR for removing diclofenac and ibuprofen at concentrations of 2, 4, 7 and 10 mg/L. However, the effects of high concentration PPCPs on the simultaneous nitrification and denitrification (SND) of MBBR supported on polyurethane sponges and the impact resistance and tolerance of this system have not been investigated.

For this reason, three typical PPCPs were selected in this study to explore the following: (i) the removal efficiency of high concentrations of PPCPs using the MBBR process on the basis of the removal of nitrogen; (ii) the inhibitory effect of high concentrations of PPCPs on the removal of organic matter and nitrogen; (iii) the toxicity of different PPCPs on the adherent biomass of bioarriers; and (iv) variation in microbial diversity and relative abundance under different concentrations and types of PPCPs. This study can promote a better understanding of the impacts of resistance and tolerance on the MBBR process on PPCPs. It

will also provide some guidance on how to degrade PPCPs during wastewater treatment.

2. Materials and methods

2.1 Experimental set-up and operation

Four cubic lab-scale MBBR reactors (L = 35 cm, W = 9.5 cm, H = 49 cm) made of plexiglas were used during room temperature in parallel for the experimental study, with an effective working volume of 12 L. According to the different types of PPCPs, the four MBBRs were named R1 (none PPCPs), R2 (sulfadiazine), R3 (ibuprofen) and R4 (carbamazepine), respectively. The 9 cm air stones were installed at the bottom of the reactor with an air flow of 0.09 m^3/h, mainly to ensure the dissolved air concentration of 5-6.5 mg/L was retained and the regime in the reactor was completely mixed. Twenty percent of the reactor's effective volume was filled with cubic polyurethane sponges known as biocarriers (purchased from Joyce Foam Pty, Australia) with a side length of 15 mm, a density of 28 kg/m^3 and an average specific surface area of 0.846 m^2/g. All the reactors were seeded with activated sludge (the initial mixed liquor suspended solids (MLSS)) was 2.8 g/L obtained from a secondary sedimentation tank at a local municipal wastewater treatment plant, located in Tianjin, China. The sponge carriers added in all MBBR reactors were acclimatized to the synthetic wastewater for 15 days before operating in continuous mode with a flow rate of 16.7 mL/min and a hydraulic retention time (HRT) of 12h.

The experimental process lasted 90 days and these were divided into three periods according to different concentrations of PPCPs. In Period I (Day 1–30), the dosages of PPCPs added in R2, R3 and R4 were 1 mg/L. Correspondingly, the concentrations of PPCPs were 2 mg/L and 5 mg/Lin Period II (Day 30–60) and Period III (Day 60–90), respectively.

2.2 Synthetic wastewater

To avoid fluctuation in the feed concentration and provide a continuous source of biodegradable organic pollutants, the experiments were conducted using synthetic wastewater composed of the following: 110–120 mg/L total organic carbon (TOC), 28–33 mg/L NH_4^+-N, 2.7–3.5 mg/L total phosphorus, 0.5–2.5 mg/L NO_3^--N and 0.02–0.11 mg/L NO_2^--N. The trace nutrient solution contained in this study was based on that used in the study by Zhang et al. (2017). Based on the components of the synthetic wastewater, the TOC/TN (C/N) ratio of the influent in the experiments was around 3.5 (Song et al., 2018). All chemicals mentioned above were purchased from Tianjin, China, and the grades were of analytical purity. Sulfadiazine, ibuprofen and carbamazepine (superior purity) were purchased from Shanghai Dibai Biotechnology Co., Ltd., the physicochemical properties of which are summarized in Table 1.

Table 1

2.3 Analytical methods

TOC of the influent and effluent was measured using a TOC analyzer (TOC-VWP, Shimadzu, Japan). Concentrations of NH_4^+-N, NO_3^--N, NO_2^--N and MLSS were characterized by standard methods (APHA, 2005). TN and SND were calculated according to the study by Song et al. (2018). Attached-growth biomass (AGBS) and volatile attached-growth biomass (VAGBS) was obtained by hand squeezing the sponge cubes and rinsing the squeezed cubes with ultrapure water (Luo et al., 2014). The calculation methods were based on what was used in our previous study (Song et al., 2018):

$$\text{TN removal efficiency} = (1 - \frac{NH_{4\,eff}^{+} + NO_{2\,eff}^{-} + NO_{3\,eff}^{-}}{NH_{4\,\text{inf}}^{+} + NO_{2\,\text{inf}}^{-} + NO_{3\,\text{inf}}^{-}}) \times 100\% \quad (1)$$

$$SND = (1 - \frac{NO_{2\,eff}^{-} + NO_{3\,eff}^{-} - NO_{2\,\mathrm{inf}}^{-} - NO_{3\,\mathrm{inf}}^{-}}{NH_{4\,\mathrm{inf}}^{+} - NH_{4\,eff}^{+}}) \times 100\% \quad (2)$$

where $NH_4^+{}_{inf}$, $NO_2^-{}_{inf}$ and $NO_3^-{}_{inf}$ are the NH_4^+, NO_2^- and NO_3^- concentrations in the influent (mg/L), $NH_4^+{}_{eff}$, $NO_2^-{}_{eff}$ and $NO_3^-{}_{eff}$ are the NH_4^+, NO_2^- and NO_3^- concentrations in the effluent (mg/L).

2.4 PPCPs analysis

2.4.1 Sample pretreatment

Solid phase extraction (SPE) served as a pretreatment method for water samples. The SPE columns were Oasis HLB (6 mL/500 mg, USA) from the Waters Company. Methanol and ultrapure water was successively employed to flush the SPE columns in order to remove impurities in the pipeline and prevent cross-contamination prior to the solid phase extraction. SPE columns must be activated before use in order to create a suitable solvent environment and remove impurities.

The detailed procedures are as follows: the SPE columns were activated by 2×3 mL methanol and 2×3 mL ultrapure water in turn, and then the water samples (adjusted by 0.1 mol/L HCl and NaOH to PH to 3.5-4.0) were passed through the columns at a flow rate of 2-3 mL/min. After all the water samples were filtered, the columns were immediately rinsed with 2×3mL ultrapure water and drained with negative pressure for 1h. After that, 4×3mL methanol was used as the elution solvent to elute the target substance under the condition of gravity and collect the eluent. The eluent was stripped with nitrogen to less than 1 mL in a water bath at 40°C. The sample was re-dissolved by methanol repeatedly eluting the side wall, and the volume was fixed to 1 mL. Finally, 0.22 um nylon filters were used to filter, and then transferred to the sample bottles for testing.

2.4.2 HPLC-MS/MS

1200 high performance liquid chromatography (HPLC) (Agilent, USA) coupled with a 6410B triple quadrupole mass spectrometer (MS) (Agilent, USA) served the quantitative analysis of PPCPs (Song et al., 2019). An Agilent Eclipse C18 (2.1 mm x 150 mm i.d., 3.5 um) chromatographic column helped to detect the three target pollutants. The column temperature was 35°C, the flow rate was 0.3 mL/min, and the injection volume was 10μL. The mobile phases and their volume ratios are shown in Table 2. An electrospray (ESI) ion source and multi-reaction monitoring (MRM) scanning method were selected for mass spectrometry analysis. The atomization pressure was 35 psi, the desolvent flow rate was 9.0 L/min, the temperature was 350°C, and the capillary voltage was 4000V. The mass spectrometric conditions of the three target pollutants are summed up in Table 3.

Table 2

Table 3

2.5 Microbial community analysis

The biofilm samples were collected from each reactor at different stages (20, 50 and 80 days) to reveal microbial characteristics. The biofilm samples collected from R1 (without PPCPs), R2 (sulfadiazine), R3 (ibuprofen) and R4 (carbamazepine) on Day 20 (1 mg/L PPCPs) were named A1, A2, A3 and A4, respectively. Similarly, the biofilm samples collected on Day 50 (2 mg/L PPCPs) were named B1, B2, B3 and B4, respectively, and the biofilm samples collected on Day 80 (5 mg/L PPCPs) were named C1, C2, C3 and C4, respectively. The microbial community of the samples were examined using high-throughput sequencing of the 16S rRNA gene. The variable regions (V3, V4) from the genomic DNA were amplified effectively via a primer system set at 341F/805R. After the extraction and quality inspection

of the sample genome DNA were completed, the PCR amplification and product purification were carried out. The Illumina MiSeq sequencing was then implemented (Thelusmond et al., 2018). The FLASH software was used to pair the double-ended sequences based on overlapping bases. Effective sequences were obtained by QIIME (Li et al., 2015). OTU clustering was carried out: (i) to unique sequence (repeat number >1) according to the 97% similarity, (ii) to remove further the chimera sequence in clustering process toward obtaining the representative sequence of OUT at last (Zhang et al., 2019).

3. Results and discussion

3.1 Removal of the selected PPCPs

The removal efficiency of PPCPs using the MBBR reactors was investigated at different concentrations. The results are shown in Fig. 1. In the first period of the experimental operation (1 mg/L PPCPs), the removal efficiencies of sulfadiazine, ibuprofen and carbamazepine were relatively high, which was 71.1±4.8%, 84.8±3.4% and 37.5±1.6%, respectively. With the increase of the PPCPs concentration in the experimental water from 1 mg/L (Day 1-30) to 2 mg/L (Day 30-60) and then 5 mg/L (Day 60-90), the overall removal efficiencies of the PPCPs by MBBRs gradually declined. In the third period of experimental operation, the removal efficiencies of sulfadiazine, ibuprofen and carbamazepine dropped to 49.6±4.7%, 63.5±6.2% and 19.7±1.2%, respectively. This was mainly due to the increased PPCPs concentration in the influent, where the toxic effect of PPCPs on microorganisms also increased. In this way the MBBR reactors were greatly impacted, and furthermore reduced the ability to degrade PPCPs (Nguyen et al., 2019).

In this entire experimental process, the average removal rates of sulfadiazine, ibuprofen and carbamazepine by reactor R2, R 3 and R4 were 61.1±8.8%, 74.9±8.8% and 28.3±7.4%,

respectively. The membrane bioreactor (MBR) under aerobic conditions was operated by Hai et al. (2011) to remove persistent micro pollutants such as carbamazepine. A low removal efficiency of (12 ± 11%) was observed during operation under aerobic conditions ((DO > 2 mg/L). A cyclic anoxic/aerobic membrane bioreactor was selected by Mariem et al. (2018) to evaluate the performance of treating a synthetic influent with a high PPCPs content, and results showed that the removal was less (36.2 ± 6.8%) for carbamazepine due to its recalcitrance. Jiang et al. (2017) evaluated micro pollutants (ng/L) removal of a hybrid MBBR-MBR system at four different HRTs (24, 18, 12 and 6 h), and the results revealed that the MBBR systems at all HRTs could effectively remove ibuprofen (> 92%), while carbamazepine showed particularly low removals (< 27%). In another study, Yu et al. (2018) investigated the removal and degradation mechanisms of sulfonamide antibiotics, and their experimental results indicated that the efficiency in removing sulfadiazine was 76.6% in a conventional aerobic submerged membrane bioreactor. In contrast, the MBBR process using the polyurethane sponges as biocarriers could remove typical PPCPs with high concentrations effectively. The removal efficiency of ibuprofen was in fact the highest, followed by sulfadiazine and then carbamazepine.

Biodegradation, adsorption and volatilization are common ways of removing PPCPs from wastewater using MBBR reactors. For the pollutants whose Henry's constant (k_H) were in the 10^{-3} and 10^{-2} range, the volatility was better (Stenstrom et al., 2010), while the values of sulfadiazine, ibuprofen and carbamazepine were all in the range of 10^{-4} and 10^{-11} (Luo et al., 2014), and volatility could be ignored . For some stable compounds that have a strong persistence, the adsorption removal method becomes significant especially with carbamazepine. However, adsorption is a short-term process, and the removal rate of

carbamazepine is low because while it is absorbed it is not degraded (Wang and Wang, 2017). Biodegradation is the main method to remove pollutants in the MBBR. For hydrophobic compounds with logD < 2.5, biodegradability is strong, such as ibuprofen, which contains powerful electron donors (-OH), and therefore it can be removed effectively (Luo et al., 2014). However, carbamazepine is more recalcitrant due to possessing the electron withdrawing group (amide: $CONH_2$) (Jiang et al., 2017). In addition, aerobic conditions are conducive for the removal of ibuprofen, while anoxic conditions make it possible to remove of carbamazepine (Thelusmond et al., 2018). Whilst the aerobic environment in the MBBR reactors were dominant, the removal rate of ibuprofen was higher (Luo et al., 2014). For sulfadiazine, biological degradation is the major mechanism used for its removal in the MBBR reactors (Yu et al., 2018). Sulfadiazine (pKa_2: 6.5) will become negatively charged when the pH > pKa_2, leading to an electrostatic repulsion between sulfadiazine and biofilms (Tambosi et al., 2010), and low $LogK_{ow}$ value of sulfadiazine ($LogK_{ow}$ < 2) will lead to the poorer adsorption capacity of bioflims (Cheng et al., 2018). Therefore, the main way to remove sulfadiazine was through biodegradation rather than adsorption.

Figure 1.

3.2 Effect of PPCPs on the performance of MBBR reactors

3.2.1 Effect of PPCPs on TOC removal in MBBRs

Fig. 2 (a) illustrates the concentration changes of TOC in four MBBR reactors during the entire experimental operation stages. As can be seen from Fig.2 (a), the removal of TOC by R1 was maintained at a high level, with an average removal efficiency of 94.7±0.7%, while the removal of TOC by R2, R3 and R4 with sulfadiazine, ibuprofen and carbamazepine had been inhibited to some extent. They achieved average removal efficiencies of 83.7±3.8%,

87.1±2.1% and 88.4±2.1%, respectively. The inhibition of ibuprofen and carbamazepine on TOC removal was slightly different, while sulfadiazine demonstrated the most inhibition on microorganisms related to the removal of organic matter.

3.2.2 Effect of PPCPs on the removal of nitrogen in the MBBRs

From Fig. 2 (b), it can be seen that the average removal efficiency of NH_4^+-N using R1 reached 97.4±1.5%, while the average removal efficiencies of sulfadiazine, ibuprofen and carbamazepine using R2, R3 and R4 were 89.4±5.2%, 83.1±6.1% and 74.1±6.7%, respectively. The average removal efficiencies of TN using R1, R2, R3 and R4 were 84.2±3.9%, 63.4±4.8%, 53.8±10.2% and 42.6±10.8%, respectively, and the corresponding SND performance was 91.0±3.6%, 74.5±6.6%, 67.7±8.4% and 59.8±10.5%, respectively (Fig. 2 (c)). Compared with reactor R1, the removal efficiency of TN in the reactors with sulfadiazine, ibuprofen and carbamazepine fell by 21%, 30% and 42%, respectively. The performance of SND declined by 17%, 24% and 31%, also respectively.

Although these PPCPs had a certain inhibitory effect on the removal of NH_4^+-N, it can be concluded from the inhibition results of TN that the degree of inhibition on denitrification was greater than that of nitrification. This phenomenon may be due to the following reasons. Firstly, as denitrifying bacteria belong to the heterotrophic bacteria (Tran et al., 2013), the self-protection mechanism of other heterotrophic bacteria in the system gradually strengthened after exposing to PPCPs, and more nutrients were absorbed to maintain survival (Li et al., 2017). Therefore, the denitrifying bacteria existing inside the biocarriers cannot be in contact with enough nutrients and consequently gradually perished. This subsequently led to a decline in the denitrification performance for the entire system. Secondly, because the removal of TN by MBBR reactors depends on the joint action of nitrifying bacteria and

denitrifying bacteria. The nitrifying bacteria outside the biocarriers metabolize swimmingly with the biocarriers moving continuously (Young et al., 2016), and the effect of mass transfer was better. While the denitrifying bacteria inside the biocarriers under the poisonousness of PPCPs cannot complete metabolism, thereby leading to a poor denitrification outcome.

3.2.3 Effect of PPCPs on biomass in the MBBRs

Fig. 2 (d) illustrates the change in the attached biomass in four MBBR reactors. At the initial stage (1-10 days) of the experimental operation, the attached biomass in all reactors decreased rapidly. This is due to the fact that the inoculated sludge and the unstable biofilms attached to the surface of the biocarriers were then discharged with the effluent. During the 10-30 days of the experiments, the microorganisms were in the rapid growth stage, indicating that they could survive and reproduce normally in this environment. With the gradual stabilization of the reactors (30~60 days), the attached biomass of the biocarriers increased gradually, and this increase indicated that the growth rate of microorganisms was higher than the death rate, which meant that many microorganisms could in fact tolerate the concentration of 2 mg/L PPCPs. With the increase in PPCPs concentration to 5 mg/L (60-90 days), the attached biomass did not change, which suggested that the environment in the system had become too harsh for microorganisms at this point in time. Their growth rate decreased and the death rate increased. As a whole (Fig. 2 (e)), the total microorganisms in R1 without any PPCPs were the largest, with an average attached biomass of 0.405±0.094g/g carrier, followed by R3 with ibuprofen, with an average attached biomass of 0.349±0.078 g/g carrier. However, the biomass in R4 and R2 reactors of carbamazepine and sulfadiazine were relatively small, with a small difference of 0.308±0.060 g/g carrier and 0.298±0.054 g/g carrier, respectively.

Figure 2.

3.3 Effect of PPCPs on microbial diversity and similarity

3.3.1 Microbial diversity analysis

Biodiversity is essential in order to improve the performance of reactors and thus biodiversity indices are considered important variables to describe these microbial communities (David et al., 2018). On average, approximately 60,000 quality filtered reads were measured per sample from Illumina sequencing. A total of 17,688 to 61,802 effective gene sequences with an average length of 450 bp was obtained from biofilm samples. The Good's coverage varied in the 0.994 and 0.998 range at 1, 2 and 5 mg/L PPCPs, suggesting that the sequences of the biofilm samples covered the majority of bacterial diversity in MBBR reactors.

According to the different sampling stages (20, 50 and 80 days), all samples were divided into three groups: A (A1, A2, A3 and A4), B (B1, B2, B3 and B4) and C (C1, C2, C3 and C4). The Venn diagrams with shared and unique OTUs depicted the differences of microbial community at 1 mg/L(A), 2 mg/L(B) and 5 mg/L(C) PPCPs (Fig. 3 (a)). The following recorded totals were 1469, 1531, and 1348 OTUs on Day 20, 50, and 80, respectively, of which 531 OTUs were shared by them, demonstrating that some bacteria had always existed in the MBBRs at all PPCPs concentrations. The phylum level mainly included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, which was consistent with previous studies (Feng et al., 2019; Li et al., 2017). The groups B and C shared the most OTUs (240, 45.20% of the total), followed by the groups A and C (228, 42.94%) and groups A and B (216, 40.67%).

The unique OTUs for biofilm samples were 494, 544 and 349 on day 20, 50 and 80, respectively, which indicated that the diversity of biofilm samples on day 50 was most

abundant. The OTUs increased from 1 mg/L PPCPs to 2 mg/L PPCPs, because the self-protection mechanism for microorganisms to cope with harsh environments had been activated (Zhang et al., 2019). When the concentration of pollutants was within a certain range, microorganisms stimulated by PPCPs produced a self-protection mechanism, and some microorganisms sensitive to PPCPs began to multiply rapidly, so the diversity of microflora displayed an increasing trend. While the number of OTUs reduced from 2 mg/L PPCPs to 5 mg/L PPCPs, this is due to it exceeding the tolerance of microorganisms for PPCPs. When its toxicity was greater than the self-protection of microorganisms, some bacteria could not withstand toxicity and disappeared, so the richness and diversity of bacteria consequently declined (Yu et al., 2019). The very different outcomes reported in this current research when compared to a previous report (Wan et al., 2017) might be attributed to the difference in influent composition, PPCPs types, microbial concentration and microbial community structures for different bioreactors.

3.3.2 Principal component analysis (PCA)

Principal component analysis (PCA) was used to visualize the microbial communities of all samples and obtain useful insights into the similarity between the MBBRs with different PPCPs as well as the change in the microbial community composition with PPCPs concentrations. As shown in Fig. 3 (b), PC1 and PC2 could account for 33.06% and 18.21% of the total community variance, respectively. At both PC1 and PC2 levels, the gene patterns in group A with the same PPCPs concentration were closely clustered, which indicated that the biological characteristics of biofilms under 1 mg/L PPCPs regardless of the types depended on the control experiment with no PPCPs in the aerobic MBBR reactors. It appears to be that when the concentration of PPCPs is lowest, the toxicity to microorganisms on

biofilms was also less and in this case, most microorganisms could survive in these three MBBR reactors with sulfadiazine, ibuprofen and carbamazepine respectively. Therefore the microbial community structures were highly similar to the control reactor without the PPCPs.

However, the PCA plots for groups B and C show a clear separation from group A, indicating that they had different microbial community structure patterns. Pollutants change the microbial community structure by affecting the metabolic pathways of microorganisms. The inhibition of low-concentration pollutants on microorganisms tended to disappear, whereas e high-concentration pollutants caused greater loss of microorganisms (Xu et al., 2016). Diverse microbial communities have different tolerance capacities to PPCPs pollutants. With the increase of PPCPs concentration, the toxicity to microorganisms also increases, which affect the metabolic activities of these microorganisms, and results in great changes in the microbial community structure and diversity (Stadler and Love, 2016). Therefore, in this study, there were significant differences between group C with a high-concentration PPCPs (5 mg/L) and group A with a low-concentration PPCPs (1 mg/L). This is consistent with the results documented in Xu et al. (2016), who indicated that the magnitude of the changes in microbial community structure observed for the lower contamination levels was always lower to that of the observed for the high level.

Furthermore, it was clearly visible that the separation between the control and pollutant-treated reactors changed depending on the types of pollutants. Taking group B as an example (the concentration of PPCPs in B2, B3 and B4 were all 2 mg/L at Day 50), the order of separation from the control was B1B2 > B1B3 > B1B4. Specifically, the reactor with sulfadiazine had the greatest difference with the control, followed by the reactors with ibuprofen and carbamazepine. The same regularity was also observed in group A and group C.

There are two reasons for this phenomenon. One is that the physical and chemical properties of different pollutants wield different effects on the microbial community structure (Pino-Otín et al., 2017). The other is that the sensitivity and tolerance of microorganisms to different pollutants are different (Thelusmond et al., 2016).

Figure 3.

3.4 Effect of PPCPs on microbial community structure

In order to further investigate the microbial community structures in the four MBBR reactors during the 90 days of operation, the 12 samples under different PPCPs concentrations and pressure types were analyzed by phylogenetic analysis of the 16S rRNA gene sequences at the phylum and genus levels (Fig. 4).

The distribution of the total bacteria at the phylum level in all biofilm samples is shown in Fig.4 (a). *Proteobacteria* and *Bacteroidetes* were the two most dominant phyla during the whole process in the four reactors, which agreed with what other studies have found (Feng et al., 2019; Song et al., 2018). The *Proteobacteria* belongs to the Gram-negative bacteria, which is related to the removal of nitrogen and can also resist PPCPs stress (Zhou et al., 2019). Previous studies have shown that *Proteobacteria* played a leading role in the removal of organic matter and nitrogen in sewage treatment, and the degradation of PPCPs (Vasiliadou et al., 2018). The phylum *Bacteroidetes* consists of gram-negative, non-spore forming and rod-shaped cells (Ng et al., 2016), and has been reported as consisting of potential degradation bacteria for PPCPs (Zhang et al., 2018a). The abundance of *Bacteroidetes* in the biofilm samples of reactor R1 without PPCPs increased gradually throughout the experiment (A1: 13.98%, B1: 22.69%, C1: 29.18%). Except for R1, the relative abundance of *Bacteroidetes* in reactor R4 with carbamazepine being the highest (A4: 13.79%, B4: 16.99%,

C4: 15.58%), followed by reactor R3 with ibuprofen (A3: 14.83%, B3: 11.14%, C3: 15.34%), and then reactor R2 with sulfadiazine (A2: 9.69%, B2: 9.68%, C2: 10.51%). According to the literature, *Bacteroidetes* are related to the degradation of carbamazepine (Thelusmond et al., 2018), but are unable to tolerate high concentrations of sulfadiazine (Li et al., 2017).

The third most abundant was *Actinobacteria*, followed by *Gemmatimonadetes* and then *Acidobacteria*. The relative abundance of *Actinobacteria*, which is a Gram- positive bacteria, decreased when more PPCPs concentrations were added in the three PPCPs reactors. Taking the R3 with ibuprofen as an example, the relative abundance of *Actinobacteria* decreased from 11.82% to 9.20% and then to 8.37% when the ibuprofen concentration rose from 1 mg/L to 2 mg/L and 5 mg/L. This indicated that *Actinobacteria* are not able to adapt to the toxicity of PPCPs. However, the relative abundance of *Gemmatimonadetes* in all MBBR reactors with added PPCPs increased, while the relative abundance of *Acidobacteria* decreased, which suggested that *Gemmatimonadetes* were better at adapting to the PPCPs pressure than the *Acidobacteria*. Where *Gemmatimonadetes* was not subjected to the effect of PPCPs biological toxicity, meanwhile, it could be speculated that *Gemmatimonadetes* did play an important role in the degradation of PPCPs as mentioned above (Yu et al., 2019).

In order to estimate varieties of and identify the dominant microorganisms in the context of pressure for different PPCPs, the sequences were analyzed at the genus level (Fig. 4 (b)). Due to the complex diversity of microbial species attached to the biofilms, it would be better to accurately describe the changes of microflora in the separate MBBR reactor containing no PPCPs, sulfadiazine, ibuprofen and carbamazepine, respectively.

For the biofilm samples of R1 without PPCPs, the relative abundance of *Sphaerotilus*, a chemoheterotrophic Gram-negative bacteria, increased from Day 20 to Day 80 (A1: 0.74%,

B1: 7.42%, C1: 15.90%). They easily adhered to and grew on the biofilms in MBBR reactors (Song et al., 2018). Due to no toxic pollutants being added in R1, its internal environment gradually becoming stabilized with the continuous operation of the experiment, coupled with some bacteria related to the removal of nitrogen gradually increasing, mainly: *Filimonas*; *Piscinibacter*; *Hyphomicrobium*; *Flavihumibacter*; *Dinghuibacter* and *Meiothermus*. However, the relative abundance of some genera triggered a downward trend, mainly for the following: *Nakamurella* (from 23.40% to 9.03%); *Thermomonas* (from 12.03% to 1.35%); *Ferruginibacter* (from 7.20% to 3.30%); and *Tolumonas* (from 3.46% to 0.35%). The reason for the decrease in the proportion of these microorganisms was their lack of competiveness, as these were inhibited by the increase in the microorganisms more suited to the environment in R1 (Wan et al., 2017).

For the biofilm samples for R2 with sulfadiazine, the same being for reactor R1, *Nakamurella* was the most abundant genus. In addition, *Methylophilus* playing an important role in the degradation of various PPCPs (Feng et al., 2019) came in second in relative abundance, and remained in rapid growth during the entire operation. In the initial period (1 mg/L sulfadiazine), the relative abundance of *Methylophilus* was only 0.5%, however, by the middle (2 mg/L sulfadiazine) it was 14.38% and in the later periods (5 mg/L sulfadiazine), the relative abundance of *Methylophilus* increased 31.29%. This suggests that microorganisms exposed to antibiotics may develop specific adaptations and could build-up stronger resistance to the antibiotics' effects (Vasiliadou et al., 2018). The third most abundant was *Sphaerotilus*, followed by *Filimonas* and then *Haliangium*, among which the relative abundance of *Filimonas* remained stable at any concentration of sulfadiazine, indicating that it was extremely insensitive to the harsh environment where sulfadiazine existed. The relative

abundance of *Sphaerotilus* rose when the sulfadiazine concentration increased (from 3.87% to 9.53%), while the abundance of *Haliangium* declined from 1 mg/L to 5 mg/L sulfadiazine being (from 4.71% to 1.32%). This outcome could be attributed to the self-defense mechanisms of the microbial group fighting against the harmful environment.

For the biofilm samples of R3 with ibuprofen, the top five most abundant genera were: *Thermomonas*; *Nakamurella*; *Sphaerotilus*; *Methylophilus* and *Filimonas*, among which *Sphaerotilus* and *Methylophilus* rose with the concentration of ibuprofen. It suggested these genera were able to adapt to the environment in R2 and were closely related to the degradation of ibuprofen. However, the growth of the above genera caused a lot of competition with and inhibitory effects towards other bacteria, namely *Thermomonas*, *Brevundimonas* and *Hydrogenophaga*. An phenomenon to emerge here is that the genus *Thermomonas* is able to: firstly, utilize organic electron donors; and secondly, reduce nitrate or nitrite to molecular nitrogen (Han et al., 2015). There was a very large proportion (17.39%) in the sample of 1 mg/L ibuprofen, however, the proportions reduced greatly in the operational process at 2 mg/L (12.07%), and 5 mg/L (8.60%) ibuprofen. This also confirmed in one study that a selective pressure of ibuprofen resulted in a shift in the microbial community structure (Davids et al., 2017). Two microorganisms, these being *Nakamurella* and *Filimonas*, remained stable and did not change significantly, indicating that they were insensitive to ibuprofen.

For the biofilm samples of R4 with carbamazepine, *Sphaerotilus* was the most abundant, but in this case, *Thermomonas* was the second most abundant. The third most abundant was *Nakamurella*, followed by *Filimonas* and then *Ferruginibacter* in last place. From Fig. 4 (b), the presence of carbamazepine had greatly changed the nature of the microbial communities.

The greatest increase in higher carbamazepine concentration, compared to the biofilm sample at 1 mg/L carbamazepine, was noted for *Sphaerotilus* (from 1.26% to 32.72%), *Methylophilus* (from 0.26% to 3.56%) and *Hyphomicrobium* (from 0.63% to 2.75%). These results including the published data by other authors demonstrated that these microorganisms were increasingly associated with the biodegradation of carbamazepine, as they appear to benefit from their removal (Thelusmond et al., 2018). Many common genera decreased in abundance at higher carbamazepine concentration levels compared to the biofilm sample at 1 mg/L carbamazepine, which mainly included *Thermomonas* (from 19.68% to 2.59%), *Deinococcus* (from 6.55% to 0.07%) and *Sediminibacterium* (from 3.11% to 0.22%). It is indicated here that carbamazepine could could help to put pressure on the development of some microorganisms which might not be able to resist the harmful environment (Zhang et al., 2019). This proves once again that the exposure to carbamazepine negatively affects the microbial community structure, and diversity (Thelusmond et al., 2016).

Figure 4.

4. Conclusions

Comparative analysis indicated that the MBBR process can effectively remove typical PPCPs from wastewater, and ibuprofen had the best removal efficiency, followed by sulfadiazine and then carbamazepine. After adding PPCPs, the removals of TOC, $NH4^{+}$-N and TN by MBBR reactors were inhibited to varying degrees, and the inhibition of denitrification was greater than that of nitrification. The MBBR reactors supported by the polyurethane sponges can withstand a typical PPCPs concentration of 2 mg/L, and thus, performance would be greatly affected when the concentration exceeded it. Consequently, the selective pressure of different types, and concentrations of PPCPs led to a variety of changes

in the microbial community structure.

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Supplementary Information

Supplementary materials associated with this article can be found in the online version.

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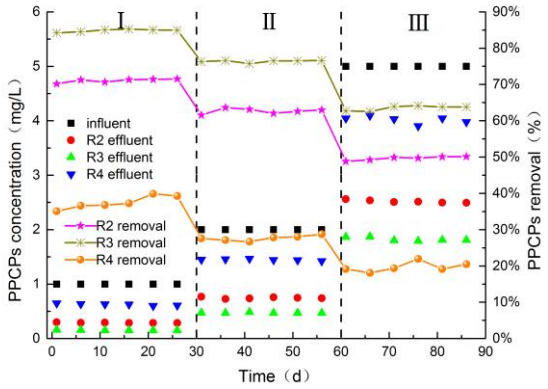


Fig. 1 Variations of PPCPs concentrations and their removal efficiencies in MBBRs: R2 (sulfadiazine), R3 (ibuprofen) and R4 (carbamazepine)

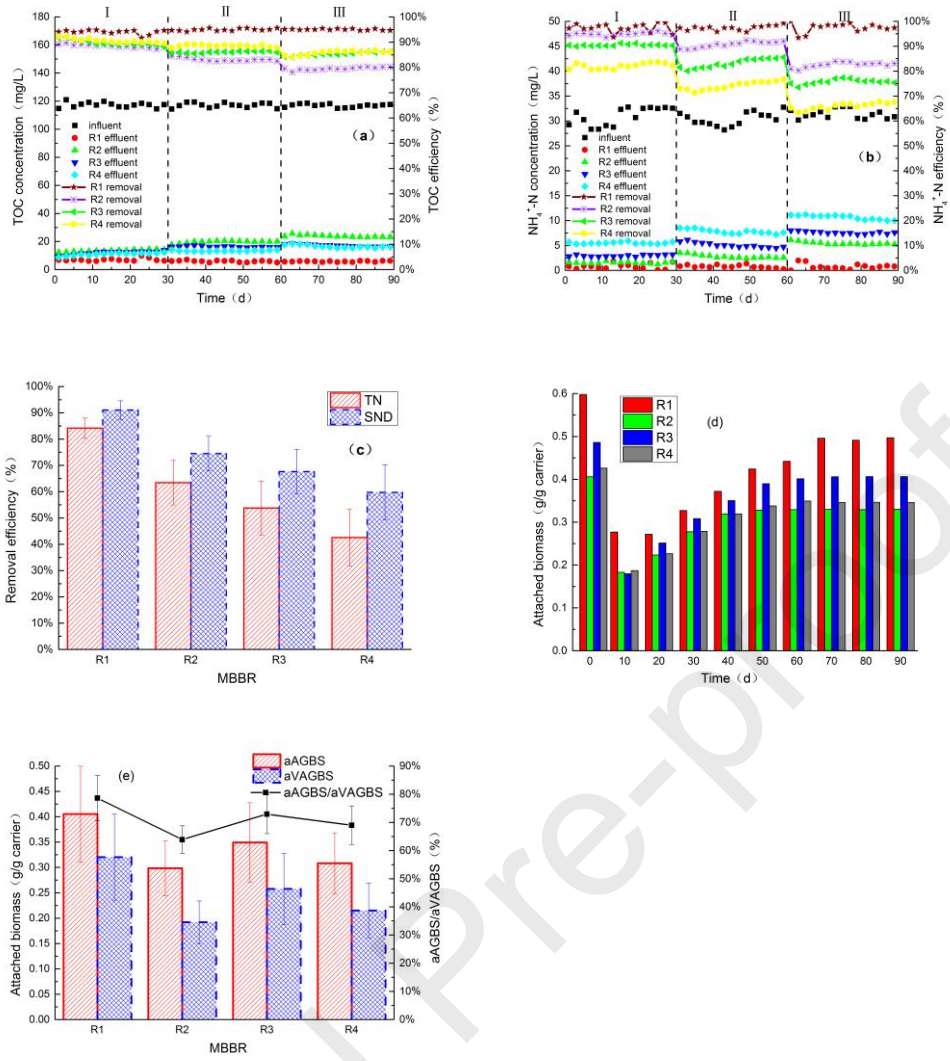


Fig. 2 Variations of TOC, NH_4^+-N, TN concentrations and their removal efficiencies as well as SND performance in MBBRs: (a) TOC, (b) $NH4^+$-N, and (c) TN and SND; The change of attached biomass (d) and average attached biomass (e) in MBBRs with different PPCPs.

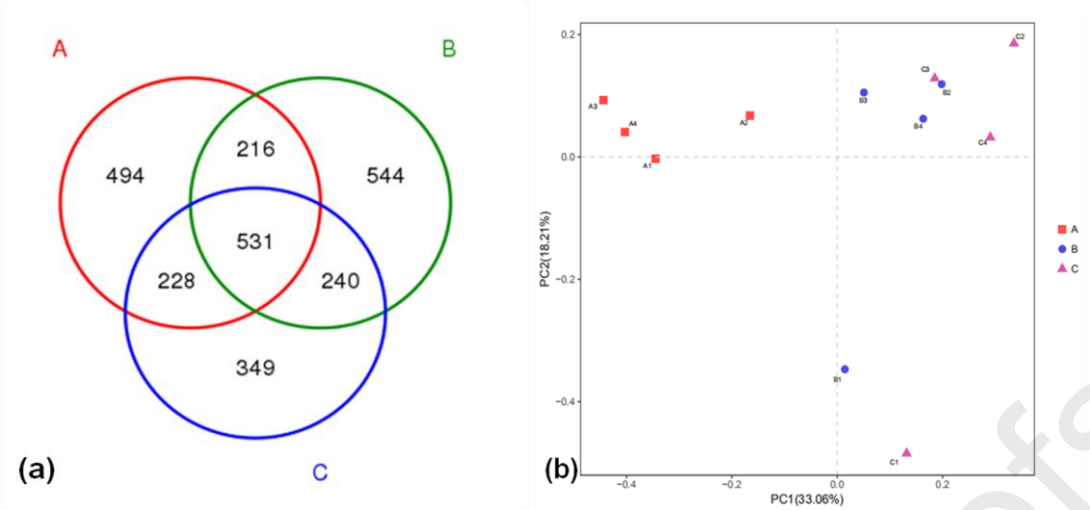


Fig. 3 Microbial diversity and similarity: (a) Venn diagram on 16S sequencing at different PPCPs concentrations; (b) Principal component analysis.

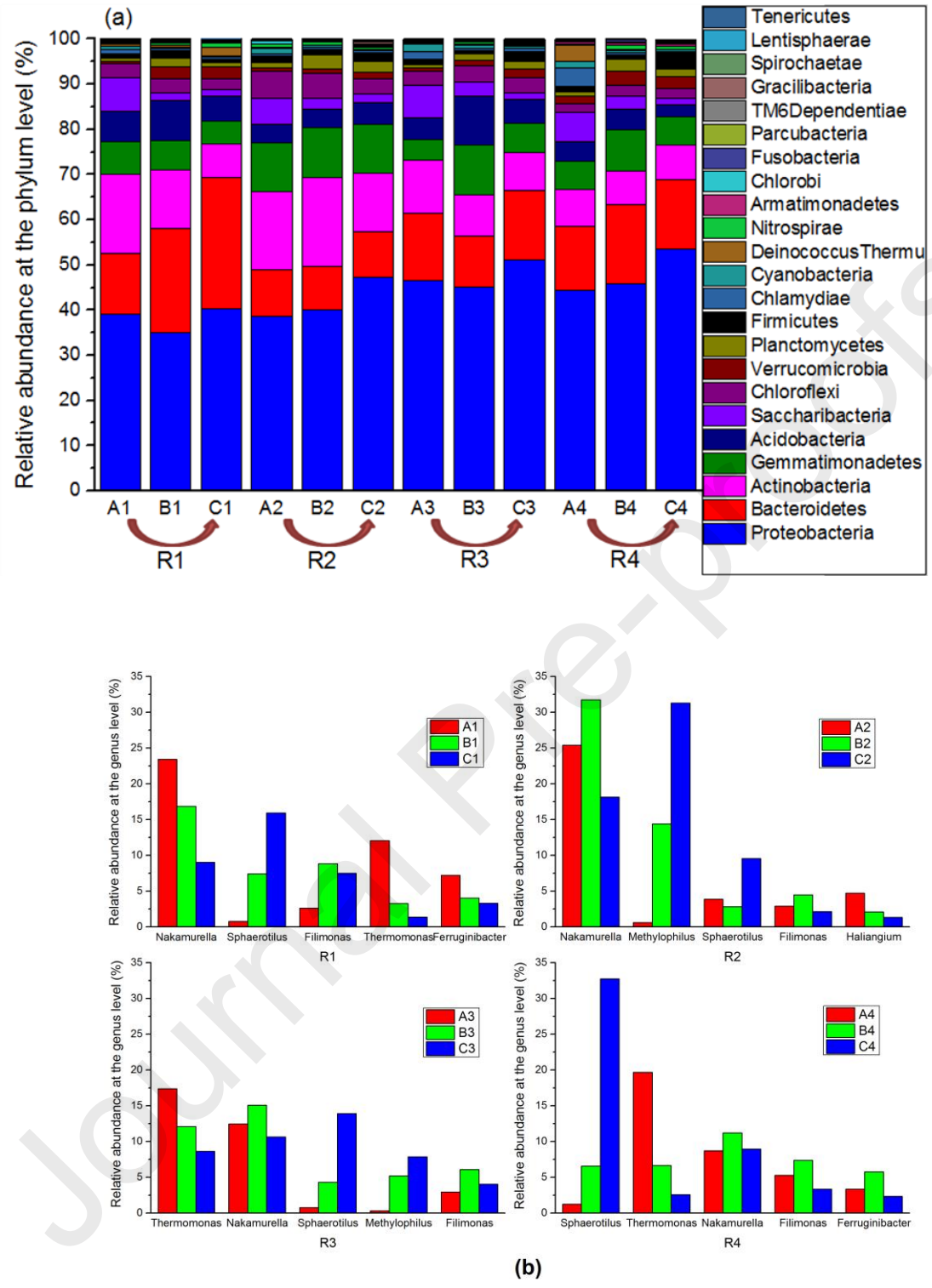
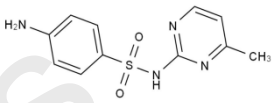
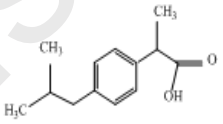
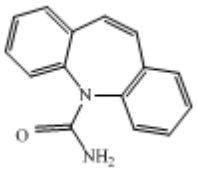


Fig. 4 Microbial composition: (a) Relative abundance at the phylum level; (b) Relative abundance of the maximum genus contained in each reactor.

Table 1

Physicochemical properties of the selected trace compounds.

Compound	CAS number	Molecular Weight (g/mol)	Log K_{OW}[a]	Log D (pH 7) [a]	Dissociation constant[a] (pKa)	Boiling point[a] (°C)	Chemical structure
Sulfadiazine ($C_{11}H_{12}N_4O_2S$)	68-35-9	264.3	0.90	na	6.5	512	
Ibuprofen ($C_{13}H_{18}O_2$)	15687-27-1	206.2	3.50	0.94	4.41	319	
Carbamazepine ($C_{15}H_{12}N_2O$)	298-46-4	236.2	1.89	1.89	13.94	411	

[a] Source: SciFinder database

https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf

na: data not available

Table 2 Chromatographic conditions of the selected trace compounds.

Compound	Mobile phase	Volume ratio
Sulfadiazine	0.1 mol/L Ammonium Formate+0.1% Formic Acid Water-Acetonitrile	20:80
Ibuprofen	0.01 mol/L Potassium Dihydrogen Phosphate-Methanol	42:58
Carbamazepine	Methanol-Ultrapure Water	56:44

Table 3 Mass spectrometric conditions of the selected trace compounds.

Compound	Ionization	Parent	Subion	De-clustering	Collision

	mode	ion	ion	voltage（DP）	voltage（CE）
Sulfadiazine	ESI+	251	108	46	34
			156	22	23
Ibuprofen	ESI-	207	161	93	19
			119	93	32.5
Carbamazepine	ESI+	237	194	50	28
			179	49	50