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Effects of different influent C/N ratios on microbial community and selected estrogens removal in a sequencing batch reactor

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ABSTRACT

Steroid estrogens are emerging environmental contaminants that have been frequently detected in the effluent of wastewater treatment plants, posing potential threats to the aquatic ecosystem and human health. In this study, the effect of different influent carbon to nitrogen (C/N) ratios (2, 5, 8, 11) on estrogens removal was investigated in sequencing batch reactors. In addition, microbial diversity, community structure and functional microbes in activated sludge were analyzed by MiSeq high-throughput sequencing. The results indicated that the removal efficiency of chemical oxygen demand risen with the increase of C/N ratios, and the values of effluent NH_4^+–N and NO_2^-–N improved, but the values of effluent NO_3^-–N reduced with the increase of C/N ratios. The removal efficiency of estrone (E_1) and 17β-estradiol (E_2) was both far higher than that of 17a-ethinylestradiol (EE_2) regardless of influent C/N ratios. The removal efficiency of EE_2 gradually enhanced with the rise of C/N, and only reached 75.69% when the C/N ratio was 8. The microbial richness in sequencing batch reactors (SBRs) with C/N ratios of 5 and 8 was higher than that of SBRs with C/N ratios of 2 and 11 but had a rather lower proportion of dominant microorganisms. Illumina sequencing showed that *Thauera*, *Tetrasphaera*, *Tessaracoccus* became dominant genera in four reactors, and the kinds of functional bacteria followed the order of $E_2 > E_1 > EE_2$. It is likely to produce the stronger synergistic elimination of estrogens by heterotrophic and autotrophic bacteria when the C/N ratio was 8. The study promotes an understanding of the impacts of influent C/N ratios on bacterial communities and estrogens removal in SBR.

Keywords: Carbon to nitrogen ratios; Microbial community; Estrogens removal; Sequencing batch reactor

1. Introduction

Steroid estrogens are typical endocrine-disrupting compounds (EDCs) that have the potential for negative effects on the endocrine systems of humans and wildlife. Among estrogens, natural estrogens such as estrone (E_1), 17β-estradiol (E_2), and estriol (E_3), synthetic estrogens such as 17a-ethinylestradiol (EE_2), have the most adverse effects found in an aqueous environment. They can cause adverse developmental and reproductive effects in aquatic organisms, such as fish, birds and mammals, even at concentrations as low as 1 ng/L [1,2]. Hanna and Cigdem reported that exposure to estrogens altered sexual development and changed the mating behavior of fish [3]. As for humans, increasing EDCs linked diseases are attracting

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public concern [4]. It was found that estrogens entered into an aqueous environment mainly through the discharge of wastewater treatment plants (WWTPs) effluent due to the incomplete elimination, and high concentrations of estrogens are frequently observed in WWTPs effluent. Zhou et al. [5] reported that the maximum effluent concentrations of E_1, E_2, E_3 and EE_2 from WWTPs were 253.8, 64.3, 61.3 and 112.4 ng/L respectively in China. Similarly, the results of Ifelebuegu [6] also indicated the higher effluent concentrations of E_1 and E_2 in Britain. Therefore, the removal of estrogens is crucial to ensure the security of aquatic environments [7–9].

Generally, estrogens targeted by the present study are either removed by direct use as electron donors for heterotrophs or via the co-metabolic degradation of ammonia-oxidizing bacteria (AOB). Heterotrophs can directly take estrogens as carbon sources and energy for growth. Currently, *Novosphingobium tardaugens* ARI-1 [10], *Sphingomonas* strain KC8 [11], *Sphingobacterium* sp. JCR5 [12], and *Pseudomonas aeruginosa* TJ1 [13] isolated from activated sludge could degrade E_1, E_2 and E_3. AOB can degrade estrogens by ammonium monooxygenase enzyme (AMO) secreted during growth. Indeed, the capability of degrading estrogens by pure AOB cultures and nitrifying activated sludge (NAS) systems has been suggested, and a positive correlation was shown between the activity of AOB and the removal of estrogens. Shi et al. [14] indicated that *Nitrosomonas europaea* was able to oxidizing E_1, E_2, E_3 and EE_2 at 200 μg/L of estrogen added in the presence of ammonia. Skotnicka et al. [15] showed that the removal efficiency of estrogens by NAS was significantly higher than that of conventional activated sludge. Hence, heterotrophs and AOBs are capable of cooperatively enhancing the elimination of estrogens [16,17]. However, Bagnall et al. [18] suggested that only heterotrophic bacteria played a predominant role in the elimination of estrogens. Thus, the results are somewhat contradictory, and further research is required to identify the contribution of heterotrophic and autotrophic bacteria to the reduction of EDCs.

It is widely acknowledged that the ratio of carbon to nitrogen (C/N) is an essential factor to affect the nitrification rate and the removal of biological nutrients. As such, it may also influence the removal of estrogens due to the variation of the microbial structure of heterotrophic bacteria and nitrifying bacteria. Previous studies mainly focused on the removal efficiency of estrogens under different organic or nitrification loads. The results of Wang et al. revealed that the concentrations of E_1 and EE_2 in the effluent membrane bioreactor (MBR) reduced with the increase of influent chemical oxygen demand (COD) concentration at the same initial concentration of steroid estrogens [19]. Moreover, higher removals of estrogens were exhibited under higher nitrification rates [20,21]. However, there is a lack of research on the relationship between C/N ratios and estrogens removal. As a result, more research is required to evaluate the influence of different influent C/N ratios on estrogen removal.

In this study, four sequencing batch reactor (SBRs) operated in parallel were constructed (1) to investigate the removal of conventional pollutants and (2) selected estrogens under different influent C/N ratios; (3) to explore the impacts of C/N ratios on the microbial community in activated sludge; (4) to evaluate the shifts of functional bacteria under different influent C/N ratios.

2. Material and methods

2.1. Experimental set-up

Four SBRs were operated in parallel under identical conditions, except for different C/N ratios influent. Each reactor was made of organic glass with an effective volume of 5 L. The SBRs were inoculated with activated sludge collected from an aerobic tank of Qingdao International Horticultural Exposition domestic WWTP located in Qingdao, China. The reactor was operated with a solid retention time of 21 d and mixed liquor suspended solids (MLSS) concentration of 2,900–3,100 mg/L. All SBRs were operated at a temperature of 20°C, and the dissolved oxygen (DO) concentration was maintained at 3.0–4.0 mg/L in the aerobic stage, and the pH ranged from 7 to 8.

Different C/N ratios of synthetic wastewater, whose composition is shown in Table 1, were prepared. Influent total nitrogen concentrations were all maintained at 60 mg/L, and influent COD concentrations were 120, 300, 480 and 660 mg/L respectively, and C/N ratios were 2, 5, 8 and 11 respectively.

After 60 d of a start-up operation, activated sludge was sampled from each SBR in order to determine the microbial community structure, then each SBR was spiked successively with E_1, E_2 and EE_2 at initial concentrations of 20 μg/L, and another 30 d operation was employed to evaluate the removal of each estrogen.

2.2. Chemicals and reagents

The estrogens used in this experiment were above 98% purity. The estrone, 17β-estradiol and 17a-ethinylestradiol were purchased from Sigma-Aldrich (USA). They were dissolved in methanol to prepare stock solutions (1,000 mg/L) and then diluted to achieve the target concentrations for the study. Methanol and acetone were purchased

Table 1
Composition of synthetic wastewater

Influent quality	Composition
Chemical oxygen demand	Sodium acetate and sodium propionate 50%, whole milk powder 34%, starch and peptone 16%
Total nitrogen	NH_4Cl
Total phosphorus nutrients	KH_2PO_4, Na_2HPO_4, KH_2PO_4, $CaCl_2$, $MgSO_4$, Na_2EDTA, $FeSO_4$, $ZnSO_4$, $MnSO_4$, $CuSO_4$

from ANPEL Laboratory Technologies (Shanghai) Inc. All the organic solvents used were of high-performance liquid chromatography grade.

2.3. Analytical methods

2.3.1. Analysis of conventional parameters

COD, NH_4^+–N, NO_2^-–N, NO_3^-–N and MLSS were measured according to Chinese National Environmental Policy Act standard methods. COD was determined by the fast digestion-spectrophotometric method, and NH_4^+–N was determined by Nessler's reagent spectrophotometry, and NO_2^-–N, NO_3^-–N were both determined by ultraviolet spectrophotometry. DO was measured by HACH-Q30d dissolved oxygen meter, and pH was measured by REX PHS-3C pH Meter.

2.3.2. Analysis of estrogens

Estrogens were extracted using C-18 solid-phase extraction disks and then analyzed by liquid chromatography–mass spectrometry (LC/MS). The slurry was centrifuged at 3,000 rpm for 4 min, and then the supernatant was filtered by a 0.45 μm glass fiber filter using a glass vacuum filtration system. C18 cartridge was activated with 10 mL methanol and 10 mL ultra-pure water before extracting, then the water samples were passed through the cartridge at a flow rate of 10 mL/min. The cartridge was washed with 10% methanol and was pressure-extracted for 30 mins. Estrogens were eluted from the cartridge with 10 mL of acetone, and the eluent was then dried under a gentle stream of nitrogen. The dry residual was dissolved in 1 mL of methanol, and the supernatant was used as the sample for measurement of LC/MS.

Waters Alliance 2690 type high-performance liquid chromatography and Micromass Platform LCZ mass spectrometer (Waters Corporation) were used for analysis. The measured ion used in SIM mode detection by LC/MS analysis was m/z 269.4 for E_1, m/z 271.2 for E_2 and m/z 295.1 for EE_2. The limit of detection was 1 μg/L for each estrogen.

2.3.3. Analysis of microbial community

Activated sludge was sampled after the 60-d operation, and was marked as S1, S2, S3, and S4 according to the order of influent C/N ratio 2, 5, 8 and 11, respectively. Then, DNA was extracted according to the instructions of the E.Z.N.A.® Soil DNA Kit (OMEGA, USA). Concentrations of the extracted DNA were measured by NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). DNA samples were stored at –20°C until use.

The microbial communities of sludge samples were explored by MiSeq high-throughput sequencing targeting hypervariable regions V3-V4 of bacterial 16S rRNA gene. Each sample was amplified in triplicates. Polymerase chain reaction products were purified and then normalized in equimolar amounts, and then were sequenced on Illumina MiSeq PE300 sequencer (Illumina, USA) in Shanghai Majorbio Bio-pharm Technology Co., Ltd., (Shanghai, China).

After sequencing, clean sequences were further analyzed by Quantitative Insights Into Microbial Ecology (QIIME). And then, they were clustered into operational taxonomic units (OTUs) at 97% sequence similarity. Finally, the taxonomy of each sequence was performed by the SLIVA database based on the RDP classifier algorithm.

3. Results and discussion

3.1. Removal of conventional pollutants

As shown in Fig. 1a, the average removal efficiency of COD was 77.69% ± 3.00%, 89.73% ± 1.09%, 92.69% ± 0.75% and 93.75% ± 0.62% when influent C/N ratios were 2, 5, 8 and 11, respectively. The removal efficiency of COD improved with the rise of C/N, which may be due to the competing for dissolved oxygen and substrate between heterotrophic bacteria and nitrifying bacteria in the activated sludge [22]. Heterotrophic bacteria gradually obtained an advantage against nitrifying bacteria with the increase of C/N ratios, which improved the removal of COD, while significantly reduced the nitrification rate [23]. On the contrary, nitrifying bacteria probably played a predominant role against heterotrophic bacteria when the C/N ratios became lower. Thus, the effluent of NH_4^+–N and NO_2^-–N had the highest concentrations, reaching 0.79 ± 0.11 and 0.18 ± 0.22 mg/L respectively, when C/N ratio was 11, then followed by C/N ratio was 8, while the effluent concentrations of them were rather lower than those with C/N ratios of 2 and 5 (Figs. 1b and c).

This reactor was not constructed for denitrification, resulting in the accumulation of NO_3^-–N. The average effluent NO_3^-–N concentration was 41.04 ± 1.64 mg/L when the C/N ratio was 11, far more than that of any other groups (Fig. 1d). This is due to the increasing proportion of nitrifying bacteria with the decrease of the C/N ratio, then producing more NO_3^-–N through nitrification.

3.2. Removal of estrogens

As shown in Fig. 2, the removal efficiency of E_1 and E_2 was rather higher than those of EE_2 no matter what the influent C/N ratios were, and the removal efficiency of E_1 was all more than 95%. E_2 was not detected in all samples. The removal efficiency of EE_2, all below 80%, was rather lower than those of E_1 and E_2. The EE_2 removal efficiency was the highest when the influent C/N ratio was 8, just reaching 75.69%. Estrogens were removed primarily by adsorption and biodegradation [17]. In four SBRs with different influent C/N ratios, sludge concentration, pH, temperature, and other factors affecting adsorption capacity were almost equivalent. As a consequence, the difference in removal efficiency was probably attributed to biodegradation. Consistent with the results of this paper, previous studies showed that nitrification sludge had a higher removal efficiency of E_1 and E_2 than those of EE_2 [6,24,25]. The reason why nitrifying bacteria was able to enhance the removal efficiency may be that nitrifying bacteria initially degraded estrogens by co-metabolism, and then heterotrophic bacteria further degraded the metabolites [26].

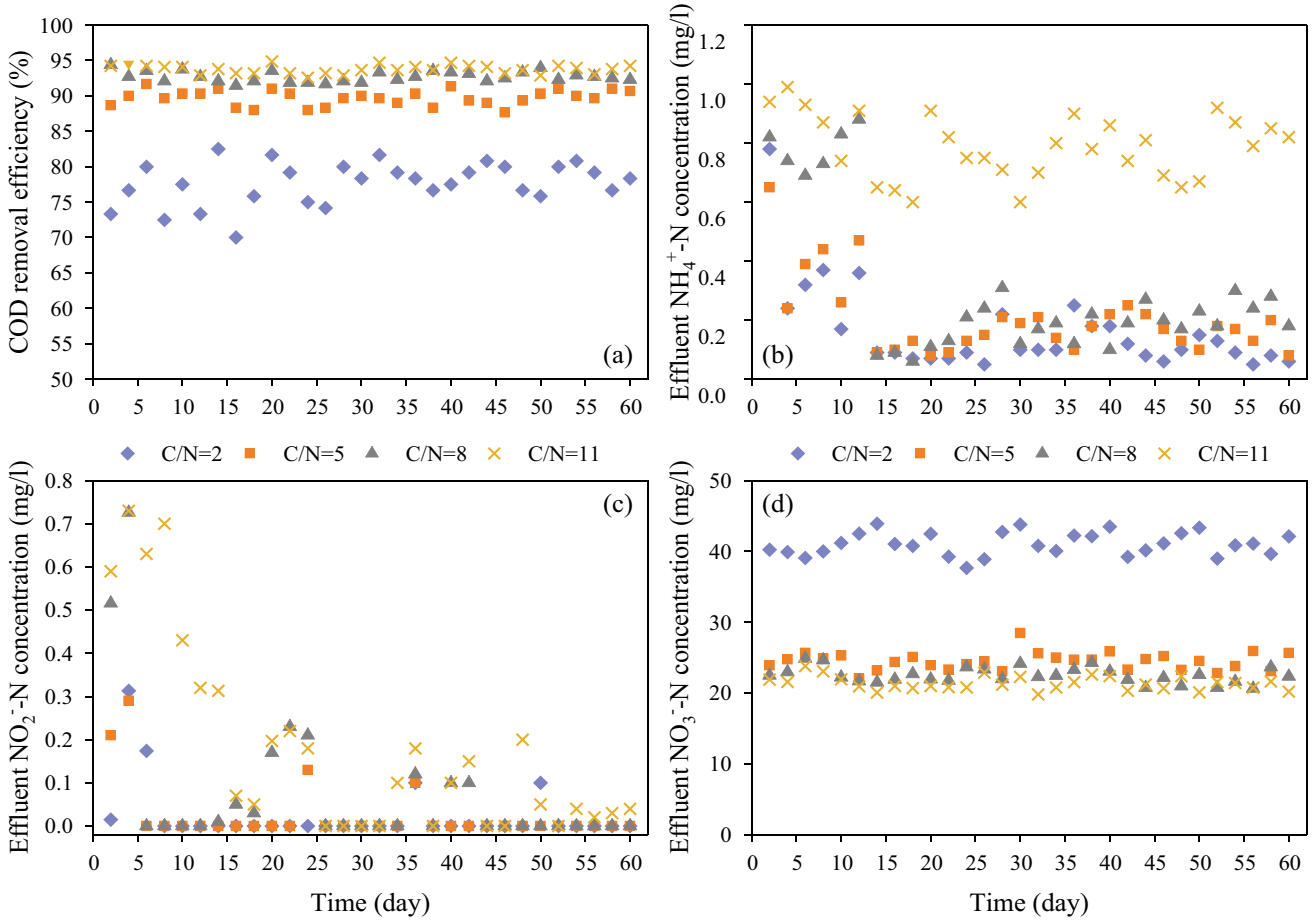

Fig. 1. Removal of conventional pollutants in SBRs with different influent C/N ratios: (a) COD removal efficiency, (b) effluent NH_4^+–N, (c) effluent NO_2^-–N, and (d) effluent NO_3^-–N.

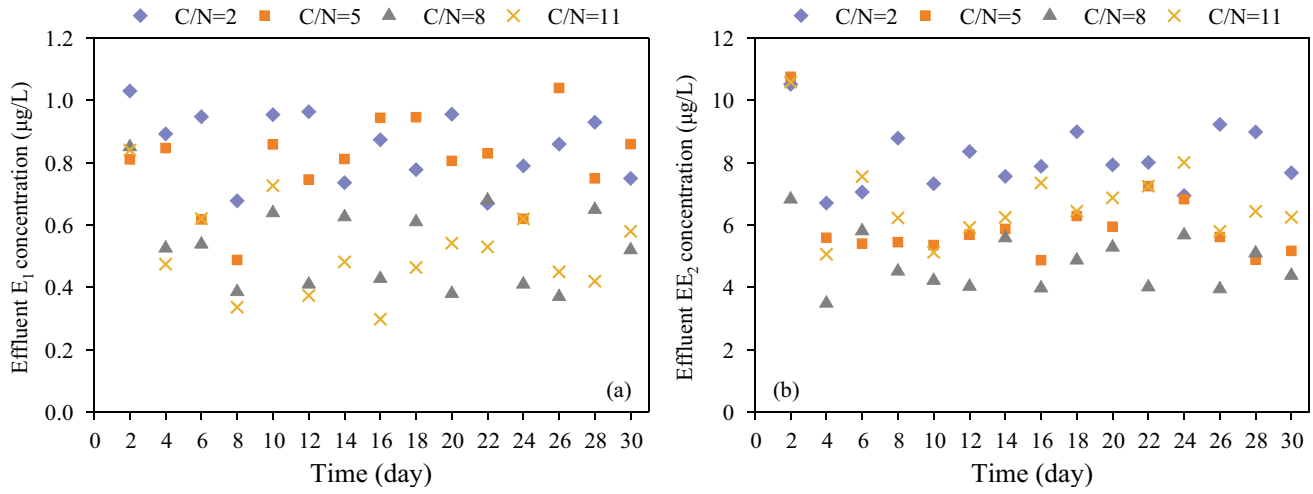

Fig. 2. Removal of estrogens in SBRs with different influent C/N ratios: (a) effluent E_1 and (b) effluent EE_2.

Table 2
Species abundance and diversity of microbial communities in the SBR

Sludge sample	Sequences number	OTU number	Shannon index	Simpson index	ACE index	Chao index	Species coverage (%)
S1	49,386	474	4.70	0.019	489.24	493.19	0.9988
S2	45,830	497	4.63	0.025	508.57	508.15	0.9989
S3	42,256	489	4.69	0.023	504.09	512.79	0.9987
S4	48,246	489	4.76	0.019	503.18	505.00	0.9989

From the functional bacteria summarized in Table 3, it was clearly observed that almost all of the bacteria had the ability to degrade E_2, which probably led to the higher removal efficiency of E_2 than those of the other two estrogens. E_2 was firstly transformed to E_1, causing the rise of E_1 [14,17]. On the other hand, the number of E_1-degrading bacteria was less than that of E_2. These two reasons may result in the lower removal efficiency of E_1 than those of E_2. Compare with E_1 and E_2, the number of EE_2-degrading functional bacteria was much less. EE_2 was generally firstly degraded by co-metabolism of *Nitrosomonas*, *Nitrosospira*, *Phyllobacterium*, *Pseudomonas* and other bacteria, and then the metabolites were further degraded by heterotrophic bacteria [16]. However, Teissier and Torre [27] stated that ethynyl groups could inhibit the activity of AMO and then invalidated the AOB co-metabolic degradation. In comparison with the molecular structure of E_1 and E_2, EE_2 contained ethynyl groups, which probably weakened the removal of EE_2. Gaulke et al. [28] showed that heterotrophic bacteria played a more significant role in the process of synergistic EE_2 removal by heterotrophic and nitrifying bacteria. In this study, the number of heterotrophic bacteria improved with the increase of C/N, while nitrifying bacteria gradually reduced under the competitive stress of heterotrophic bacteria. Thus, the removal efficiency of EE_2 enhanced with the rise of the number of heterotrophic bacteria. However, the co-metabolism of nitrifying bacteria was greatly weak due to overwhelmingly suppression by heterotrophic bacteria when influent C/N ratio was 11, then causing the removal efficiency of EE_2 reducing in comparison with that of influent C/N ratio 8.

3.3. Variations of bacterial diversity and community structure

3.3.1. Bacterial diversity

The sludge samples from four SBRs (S1, S2, S3, S4) were sequenced by using the MiSeq platform. The results are shown in Table 2, and 49,386; 45,830; 42,256 and 48,246 sequences were obtained, respectively, then the optimized sequences were clustered under 97% similarity, and 474, 497, 489, 489 OTU were acquired, respectively. ACE and Chao index reflected the richness of microbial community, while Shannon and Simpson's index represented the diversity of the microbial community.

As can be seen from Table 2, both ACE and Chao index in S2 and S3 were higher than those in S1 and S4, revealing that the microbial richness in S2 and S3 were higher than those of S1 and S4. Both heterotrophic bacteria and nitrifying bacteria obtained a suitable environment for growth when the influent C/N ratios were 5 and 8, while only one certain kind of microorganism was in better growth when influent C/N ratios were 2 or 11. Moreover, both ACE and Chao indexes changed slightly, suggesting that C/N ratios had little effect on the microbial community richness. It is in good agreement with the results of Zhao et al. [29]

Table 3
Contents of some functional bacteria in sludge samples with different influent C/N ratios

Functional bacteria		OTUs				Degradability	References
Phylum	Genus	S1	S2	S3	S4		
Proteobacteria	*Brevundimonas*	927	36	74	31	Conversion of E_2 to E_1	[32]
	Phyllobacterium	49	21	22	17	Degradation of E_1, E_2, E_3 and co-metabolic degradation of EE_2	[33]
	Nitrosomonas	150	92	82	75	Co-metabolic degradation of E_1, E_2, E_3, and EE_2	[14]
						Co-metabolic degradation of EE_2	[28]
	Nitrosospira	138	120	124	105	Co-metabolic degradation of E_1, E_2, EE_2	[28]
	Denitratisoma	69	180	30	18	Co-metabolic degradation of E_1 and E_2	[34]
	Pseudomonas	5	21	103	29	Degradation of E_1, E_2, E_3 and co-metabolic degradation of EE_2	[33]
	Bdellovibrio	173	122	101	142	Degradation of EE_2	[35]
Actinobacteria	*Mycobacterium*	29	14	15	20	Conversion of E_2 to E_1	[36]
	Nocardioides	136	122	51	29	Conversion of E_2 to E_1	[30]
Bacteroidetes	*Flavobacterium*	6	7	47	65	Conversion of E_2 to E_1	[30]
Firmicutes	*Staphylococcus*	6	5	7	6	Conversion of E_2 to E_1	[36]

who found that different ammonia nitrogen loads also had no significant effect on microbial community richness in MBRs. Shannon indexes of S1 and S4 were higher than those of S2 and S3, while Simpson indexes of S4 and S1 were lower than those of S2 and S3, indicating that the dominant population accounted for a larger proportion of the total biomass in S1 and S4 than that in S2 and S3.

3.3.2. Bacterial community composition at phylum level

It can be observed from Fig. 3 that the microorganisms in the four sludge samples mainly included Proteobacteria, Bacteroidetes, Actinobacteria, *Candidatus Saccharibacteria*, *Chloroflexi*, *Parcubacteria*, *Nitrospirae* and *Acidobacteria*. Three phylum groups with higher abundance were Proteobacteria, Bacteroidetes and Actinobacteria, which covered nearly 70% of the total bacteria, among which Proteobacteria was the most abundant and its abundance in four reactors all reached more than 30%. The abundance of Bacteroidetes was higher in S2 and S3 than that in S1 and S4, reaching 19.96% and 20.45% respectively. On the contrary, the abundance of Actinobacteria was lower in S2 and S3 than that in S1 and S4. Yu et al. [30] isolated 14 kinds of E_2-degrading bacteria from the activated sludge, which belonged to Proteobacteria, Bacteroidetes, and Actinobacteria phylum respectively. As a consequence, it was suggested that estrogen-degrading bacteria mostly distribute in these three phyla.

3.3.3. Bacterial community composition at genus level and contents of functional bacteria

In order to further clarify the variation of the bacterial community, the genera with the relative abundance of more than 1% at the genus level are illustrated in Fig. 4, among which the bacterial population of higher abundance was as follows: *Thauera*, *Tetrasphaera*, *Tessaracoccus*, *Candidatus_Microthrix*, *Ornithinibacter*, *Micropruina*, *Candidatus_Competibacter*, *Acidovorax*, *Nitrospira*, *Ferruginibacter*, *Arenimonas*, *Dokdonella*, *Microlunatus*, *Brevundimonas*, *Propioniciclava*, *Nitrosomonas*, *Fluviicoccus*. *Thauera*, responsible for degrading organic matters and denitrifying, was the most abundant genus in every reactor. This phenomenon agreed with a previous study that a large number of *Thauera* was determined in active sludge [31]. In this study, the number of *Thauera* bacteria in S2 and S3 was significantly more than those in S1 and S4. *Tetrasphaera*, *Ornithinibacter*, *Micropruina*, *Candidatus_Competibacter*, and *Microlunatus*, whose jobs were to degrade organic matters, enhanced with the rise of influent C/N ratios. Whereas *Tessaracoccus*, *Candidatus_Microthrix*, *Acidovorax*, *Arenimonas*, *Brevundimonas*, and *Ferruginibacter*, whose jobs were to remove nitrogen, reduced with the increase of influent C/N ratios.

Estrogens-degrading functional bacteria and their contents are summarized in Table 3, it was indicated that *Brevundimonas*, *Mycobacterium*, *Nocardioides*, *Flavobacterium*, *Staphylococcus* were able to convert E_2 to E_1 directly, and *Phyllobacterium*, *Nitrosomonas*, *Nitrosospira*, *Denitratisoma*, *Pseudomonas* were capable of degrading E_1 and E_2 by co-metabolism, and *Phyllobacterium*, *Nitrosomonas*, *Nitrosospira*, and *Pseudomonas* could degrade EE_2 by co-metabolism. In this study, the kinds of functional bacteria followed the trend of $E_2 > E_1 > EE_2$, which led to the highest removal efficiency of E_2, then followed by E_1, EE_2 (Fig. 2).

Nitrosomonas and *Nitrosospira*, two kinds of AOBs, were the primary bacteria performing estrogenic co-metabolic degradation. Their numbers reduced with the rise of influent C/N ratios due to the disadvantage in the competition against heterotrophic bacteria. Khunjar et al. [16] discovered that AOB biotransformed EE_2 first, and then heterotrophs mineralized EE_2 and EE_2-derived metabolites generated by AOB. Thus, the elimination of EE_2 required

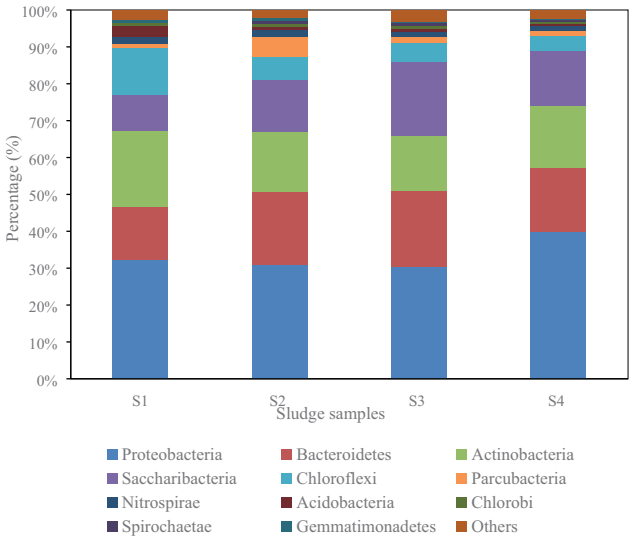

Fig. 3. Community composition at phylum level of different sludge samples.

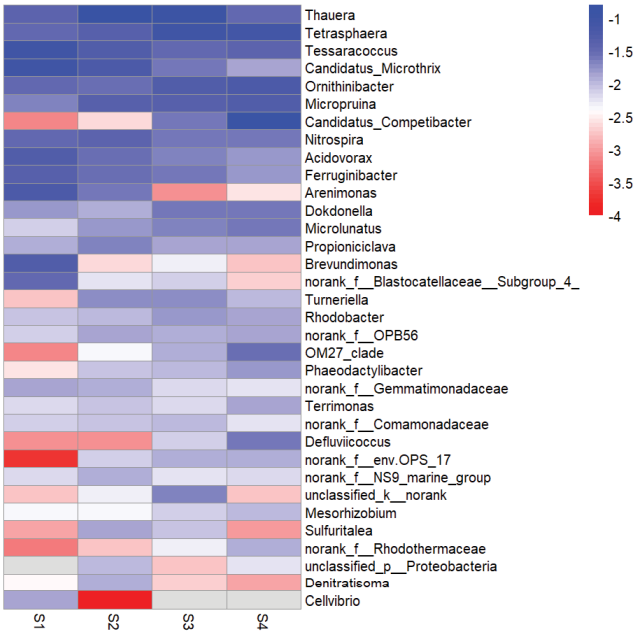

Fig. 4. Community composition at genus level of different sludge samples.

the collaboration of AOB and heterotrophs, and it was suggested from this study that both AOB and heterotrophs played a larger role when influent C/N ratio was 8.

4. Conclusion

This study investigated the effect of influent C/N ratios on microbial community and selected estrogens removal in SBRs. It appeared that there was better removal efficiency for conventional pollutants when C/N ratios were more than 5 without taking into account denitrification. In comparison with EE_2, the removal efficiency of E_1 and E_2 was both far higher than those of EE_2 regardless of C/N ratios, whose removal efficiency just reached 75.69% when the influent C/N ratio was 8. The microbial richness in SBRs with C/N ratios of 5 and 8 was similar but higher than that in the other two SBRs. *Thauera*, *Tetrasphaera*, *Tessaracoccus* all became dominant genera in four reactors, and the kinds of functional bacteria followed the order of $E_2 > E_1 > EE_2$. It is likely to produce stronger synergistic estrogens elimination by heterotrophic and autotrophic bacteria when the C/N ratio was 8.

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