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

Bingchen Zhao, Jie Lan, Dong Chen*, Linlin Li

School of Environmental and Municipal Engineering, Qingdao University of Technology, Qingdao 266033, China, Tel. +86 13969775065; email: chendong_cai@163.com (D. Chen), Tel. +86 13791997980; email: 158839819@qq.com (B.C. Zhao), Tel. +86 17860826192; email: lanjiechn@163.com (J. Lan), Tel. +86 17854267055; email: 1311880703@qq.com (L.L. Li)

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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

* Corresponding author.
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 E1, E2, E3, and EE2 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 E1 and E2 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], Sphingobacterium sp. JCR5 [12], and Pseudomonas aeruginosa TJ1 [13] isolated from activated sludge could degrade E1, E2, and EE2. AOB can degrade estrogens by ammonium monoxygenase 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 E1, E2, and EE2 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 E1 and EE2 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 E1, E2, and EE2 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-estadiol and 17α-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

<table>
<thead>
<tr>
<th>Influent quality</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td>Chemical oxygen demand</td>
<td>Sodium acetate and sodium propionate 50%, whole milk powder 34%, starch and peptone 16%</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>NH4Cl</td>
</tr>
<tr>
<td>Total phosphorus nutrients</td>
<td>KH2PO4, Na2HPO4, KH2PO4, CaCl2, MgSO4, Na2EDTA, FeSO4, ZnSO4, MnSO4, CuSO4</td>
</tr>
</tbody>
</table>
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$_3$–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$_3$–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$_2$, 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$_3$–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$_2$. 
Contents of some functional bacteria in sludge samples with different influent C/N ratios

Table 3

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

From the functional bacteria summarized in Table 3, it was clearly observed that almost all of the bacteria had the ability to degrade E1, which probably led to the higher removal efficiency of E1 than those of the other two estrogens. E2 was firstly transformed to E1, causing the rise of E1 [14,17]. On the other hand, the number of E1-degrading bacteria was less than that of E2. These two reasons may result in the lower removal efficiency of E1 than those of E2. Compare with E1 and E2, the number of EE2-degrading functional bacteria was much less. EE2 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 E1 and E2, EE2 contained ethynyl groups, which probably weakened the removal of EE2. Gaulke et al. [28] showed that heterotrophic bacteria played a more significant role in the process of synergistic EE2 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 EE2 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 EE2, 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

<table>
<thead>
<tr>
<th>Functional bacteria</th>
<th>Genus</th>
<th>OTUs</th>
<th>Degradability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria</td>
<td>Brevundimonas</td>
<td>927</td>
<td>Conversion of E1 to E2</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Phyllobacterium</td>
<td>49</td>
<td>Degradation of E1, E2, E3, and co-metabolic degradation of EE2</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>Nitrosomonas</td>
<td>150</td>
<td>Co-metabolic degradation of E1, E2, E3, and EE2</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>Nitrososira</td>
<td>138</td>
<td>Co-metabolic degradation of E1, E2, E3</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Denitratisoma</td>
<td>69</td>
<td>Co-metabolic degradation of E1, E2, E3</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas</td>
<td>5</td>
<td>Degradation of E1, E2, E3, and co-metabolic degradation of EE2</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>Bdellovibrio</td>
<td>173</td>
<td>Degradation of EE2</td>
<td>[35]</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Mycobacterium</td>
<td>29</td>
<td>Conversion of E2 to E3</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>Nocardoides</td>
<td>136</td>
<td>Conversion of E2 to E3</td>
<td>[30]</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Flavobacterium</td>
<td>6</td>
<td>Conversion of E2 to E3</td>
<td>[30]</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Staphylococcus</td>
<td>6</td>
<td>Conversion of E2 to E3</td>
<td>[36]</td>
</tr>
</tbody>
</table>
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, whereas 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. coli 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, Tesseracoccus, Candidatus_Microthrix, Ornithinibacter, Micropruina, Candidatus_Competibacter, Acidovorax, Nitrospira, Ferruginibacter, Arenimonas, Dokdonella, Microlunatus, Brevundimonas, Propionibacteria, Nitrosonomas, Chloroflexi, Phyllobacterium. These genera are likely responsible for degrading organic matters and denitrifying, which 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 Tesseracoccus, 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, Nocardides, Flavobacterium, Staphylococcus were able to convert E1 to E2 directly, and Phyllobacterium, Nitrosonomas, Nitrospira, Denitratisoma, Pseudomonas were capable of degrading E2 and E3 by co-metabolism, and Phyllobacterium, Nitrosonomas, Nitrospira, and Pseudomonas could degrade EE2 via co-metabolism. In this study, the kinds of functional bacteria followed the trend of E1 > E2 > EE2, which led to the highest removal efficiency of E3, then followed by E2, EE2 (Fig. 2).

Nitrosonomas and Nitrospira, 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 to E1 first, and then heterotrophs mineralized E1, and EE-derived metabolites generated by AOB. Thus, the elimination of EE2 required...
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 EE2, the removal efficiency of E1 and EE2 was both far higher than those of EE1, 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 E2 > E1 > EE2.

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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