Influence of operating parameters on the fate and removal of three estrogens in a laboratory-scale AAO system
Zhaohan Zhang, Yujie Feng, Hui Su, Lijun Xiang, Qiuyan Zou, Peng Gao and Peirong Zhan

ABSTRACT
A laboratory-scale anaerobic-anoxic-oxic (AAO) process was constructed to investigate the influence of hydraulic residence time (HRT) and sludge retention time (SRT) on the removal and fate of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), and their removal mechanisms in a biological treatment system. In an HRT range of 5–15 h, the highest removal efficiencies for E1, E2 and EE2 were obtained at an HRT of 8 h, with values of 91.2, 94.6 and 81.5%, respectively. When the SRT was increased from 10 to 20 d, all three estrogen removal efficiencies stayed above 80%, while the optimal SRT for each estrogen was different. The contribution of each tank for removal of the three estrogens was in the order of aerobic tank > anoxic tank > anaerobic tank. The optimal HRT and SRT for the removal of both the three estrogens and nutrients were 8 h and 15 d, respectively. At this condition, respectively, about 50.7, 70.1 and 11.3% of E1, E2 and EE2 were biodegraded, 28.8, 17.2 and 50% were accumulated in the system, 8.3, 5.4 and 17.3% were discharged in the effluent, and 12.2, 7.3 and 20.3% were transported into excess sludge. It indicated that biodegradation by sludge microorganisms was the main removal mechanism of E1 and E2, while adsorption onto sludge was the main mechanism for EE2 removal.

Key words | anaerobic-anoxic-oxic (AAO) process, estrogens, hydraulic residence time (HRT), removal mechanisms, sludge retention time (SRT)

INTRODUCTION
Estrogens are endocrine-disrupting chemicals that can cause adverse implications on reproductive and sexual systems in fish, wildlife and humans (McAdam et al. 2010). Natural estrogens, including 17β-estradiol (E2) and estrone (E1), and synthetic estrogens, such as 17α-ethinylestradiol (EE2), the main active component of the contraceptive pill, are mainly discharged to sewers in conjugated forms in urine by humans and livestock. Their primary pathways into the aquatic environment are via sewage treatment plant (STP) effluent and runoff from farmland. The reported estrogen concentrations in influent from STPs range from 7.3 to 197 ng/L (E1), 4.9 to 48 ng/L (E2) and <0.2 to <11 ng/L (EE2), which are removed within STPs with removal efficiencies ranging from 10 to 100% for E1, 39.6 to 100% for E2, and 0 to >98% for EE2 (Liu et al. 2013). Estrogen contents in natural water are much lower, with concentrations ranging from 0.8–3.9 ng/L for E1, 0.8–3.6 ng/L for E2 and nd–3.5 ng/L for EE2 (Lei et al. 2009). Although their concentrations are very low, they can cause negative effects to aquatic organisms, including vitellogenin induction, feminization, secondary sexual characteristics and abnormal development of gonads (Jobling et al. 2002).

As one of the most popular biological nutrient removal techniques, the anaerobic-anoxic-oxic (AAO) process is widely used for various wastewater treatments, such as municipal sewage, coke wastewater, piggery wastewater, etc. (Rajagopal et al. 2011). Satisfactory results were obtained for conventional index (chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) and ammonium nitrogen (NH₄-N)) removal by adjusting operating parameters, yet less attention was payed to micropollutants removal in this process. Li et al. (2011) reported that E2 was not detected in effluent and about 80% of EE2 was removed after AAO treatment, with adsorption and biodegradation as the main removal mechanisms. Zeng et al. (2015) found that the optimal SRT for estrogen and nutrient removal is...
removal was 20 days. However, few studies focused on removal mechanisms for estrogen at different HRT and SRT, and the contribution of each treatment unit for estrogen removal in biological processes.

In the biological treatment system, estrogen could be removed through three major pathways: volatilization, biodegradation and adsorption, etc. The low vapor pressure of the three estrogens \((1.06 \times 10^{-9} - 1.89 \times 10^{-5} \text{ Pa})\) implied that they were not easily volatilized under normal pressure and temperature conditions, and their loss in the biological system through volatilization was quite limited (Zhang et al. 2011). Previous studies have indicated that activated sludge had a high biodegradation efficiency for estrogens under oxic and anaerobic conditions, depending on estrogen type, microbe species and operating conditions (Shi et al. 2004; Czajka & Londry 2006). The high \(\log K_{ow}\) of the estrogens \((3.1 - 4.15)\) indicated that they could be removed from the aqueous phase by adsorption onto various adsorbents such as activated sludge, carbon nanotubes etc (Clara et al. 2004). Therefore, adsorption and biodegradation by activated sludge could be considered as the main removal mechanism for estrogens in a biological treatment system. However, which process is the dominant mechanism is still contested for estrogens with different characteristics. Hence, the removal mechanism and fate of estrogens in a biological treatment system need to be further investigated.

The objectives of this study were: (1) to evaluate the influence of hydraulic retention time (HRT) and sludge retention time (SRT) on three estrogens and nutrient removal in the AAO process; and (2) to assess the fate and removal mechanisms of estrogens during biological treatment by calculating mass balance.

**MATERIAL AND METHODS**

**Experimental set-up**

Experiments were carried out in a laboratory-scale AAO system as shown in Figure 1, which consisted of a 1-L anaerobic tank, a 1-L anoxic tank, a 3-L oxic tank and a settling tank. All reactors were made of polyethylene. Stirrers provided mixing in the anaerobic and anoxic tanks to keep biomass in suspension. Aeration was provided in the oxic tank by an air pump and stone diffusers. The influent, nitrate recirculation and sludge recycled flow were all controlled by peristaltic pumps. The circulation ratio for sludge and mixed liquor were 1.0 and 1.4, respectively.

Synthetic wastewater modeled as sewage was prepared with sucrose, sodium acetate and soluble starch as carbon source, and with ammonium nitrate and monopotassium phosphate as nitrogen and phosphorus sources. The water quality of the synthetic influent was as follows: pH 6.5, COD 470 mg/L, biochemical oxygen demand (BOD\(_5\)) 250 mg/L, NH\(_3\)-N 55 mg/L, suspended solids (SS) 250 mg/L. The estrogen concentration was set at a lower microgram per liter level, due to some industry wastewater, such as contraceptive factory or dairy lagoon wastewater containing much higher levels of estrogen than natural water. The three estrogens were all added at approximately 15.0 \(\mu\)g/L. Seeding sludge was collected from an aeration tank at Taiping STP in Harbin, China, which was concentrated to approximately 10,000 mg/L by settling; it was gray with many flocs.

The AAO system was operated at room temperature. Dissolved oxygen in the anaerobic, anoxic and oxic tanks was measured in the range of 0–0.34, 0–0.61 and 3.53–5.95 mg/L with a dissolved oxygen meter (HI9146,
HANNA Instruments, Woonsocket, RI, USA). Mixed liquor suspended solids (MLSS) in the system was about 4,000 mg/L. To study the effect of HRT on estrogen removal in the AAO system, HRT was set at 5, 8, 12 and 15 h by altering the influent flow rate with an SRT of 15 d, and the operating time for each HRT was about 1 month. For effect of SRT, SRT was adjusted to 10, 15 and 20 d by changing the amount of excess sludge from the system with an HRT of 8 h, and it lasted for about 2 month for each SRT. The system was continuously run for 10 months. Estrogen concentrations in each operating condition were expressed as a mean value and standard deviation.

Sampling and extraction

Five kinds of sample, including influent and effluent from the AAO system, mixed liquors from the anaerobic, anoxic, and oxic tanks were sampled once every 2 days for routine index analysis, and every 1 week for estrogen analysis. All samples were immediately centrifuged at 4,000 rpm for 20 min and then 10 and 50 mL supernatant were taken as stock solution for further routine indexes and estrogen analysis, respectively. Estrogens were extracted with a solid phase extraction cartridge (ENVI, Sigma-Aldrich, St Louis, MO, USA; C18, 500 mg/6 mL), which was sequentially conditioned with 5 mL methanol, 5 mL ethyl acetate and 15 mL of HCl solution (pH = 3) at a flow rate of 1 mL/min (Zhang et al. 2011). Then, samples (50 mL) acidified to 3.0 with 0.5 mol/L HCl, were percolated through cartridges at a flow rate of 8 mL/min, which was then washed with 10 mL of ultrapure water-methanol (V:V; 9:1). Cartridges were dried under vacuum for 1 h to remove water and were eluted with 10 mL of ethyl acetate into a 15 mL glass bottle at a flow rate of 1 mL/min. After dewatering with anhydrous sodium sulfate, extracts were blown dry with a gentle stream of nitrogen at 40 °C in a thermostatic water bath. Finally, the residue was re-dissolved into 0.2 mL acetonitrile for estrogen analysis.

After freeze-drying the centrifuged sludge, 1 g dry sludge was weighed and extracted with 8 and 6 mL methanol and then with 6 mL acetone, twice (Ternes et al. 2002). For each step, sample slurry was treated by ultrasound for 10 min, and then centrifuged at 3,000 rpm for 5 min. Four parts of solvent supernatants were mixed together and then evaporated to dryness with a gentle nitrogen stream. Residue was dissolved in 0.5 mL methanol, and diluted to 100 mL with ultrapure water. Then, the extraction procedure was the same as previously described for the aqueous phase.

Analytical methods

Conventional water quality indexes, such as COD, MLSS, NH4-N, TN and TP were measured following APHA Standard Methods (APHA 1998). Total organic carbon (TOC) was determined using a TOC analyzer (Shimadzu Model TOC-V, Kyoto, Japan).

Concentrations of the three estrogens (E1, E2 and EE2) were analysed by high performance liquid chromatography equipped with UV light detector (SPD-10A VP, Shimadzu, 280 nm applied) and an extend-C18 reversed-phase column (Shimpack VP-ODS, 150 × 4.6 mm, Shimadzu, Kyoto, Japan; Feng et al. 2010). The mobile phase was acetonitrile and water (50:50, v/v) with a constant flow rate of 1 mL/min. The column temperature was 25 °C and the pressure was about 120 bars. The injection volume was 20 μL. The peak time for E1, E2 and EE2 was 5.45 min, 4.35 min and 4.97 min, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were determined from a signal-to-noise ratio of 3:1 and 6:1, respectively, with an instrumental LOD of 0.04–0.07 mg/L and an LOQ of 0.08–0.14 mg/L for the three estrogens. Three replicated standard samples containing 1 μg/L of the three estrogens were used to determine the recovery and standard deviation. The mean recoveries for all three estrogens were higher than 70% in water samples. The relative standard deviations were 5–11% for water samples.

RESULTS AND DISCUSSION

Effect of HRT on the removal of three estrogens

Figure 2(a) shows the overall removal efficiencies of three estrogens in the system and percentage removal by each removal unit. When HRT was increased from 5 to 15 h, the total removal efficiencies of E1, E2 and EE2 all first increased and then decreased, with the largest removal rate of 91.2, 94.6 and 81.5%, respectively, at an HRT of 8 h, indicating that biological treatment was an efficient pathway for estrogen removal from wastewater. Too short an HRT resulted in less contact time for estrogen and microbes, leading to insufficient degradation of estrogen and its increase in effluent. However, too long an HRT resulted in too long an aeration time for the sludge, leading to self-dissolution of the activated sludge, which affected the settling property and degradation activity of microorganisms. Xu et al. (2012) found similar results when removal rates in the biological step were 23–99% for E1.
and 13–97.4% for E₂ under conditions of HRT at 13 h and SRT 11 d. There might be several reasons for relatively high estrogen removal efficiencies in the present study. First, full-scale WWTPs are always affected by many unexpected factors such as HRT, SRT, flow rate stability, temperature variations, carbon load and floc size, while a laboratory-scale AAO system can be well controlled and supplied with a stable environment for bacteria growth. Second, a broad scope of redox conditions for estrogen degradation can be provided through the combination of anaerobic, anoxic and aerobic processes. Finally, synthetic wastewater contains less other toxic pollutants and more nutrients than raw wastewater, which would benefit bacteria growth.

Removal percentages for the anaerobic, anoxic and aerobic reactors were in the range of 3.1–26.3%, 14.9–31.0% and 12.1–51.1% for E₁, 6.4–33.4%, 6.5–39.7% and 13.9–71.6% for E₂, 13.0–28.3%, 6.7–35.9% and 14.2–51.4% for EE₂, respectively. Thus, compared with anaerobic and anoxic microbes, aerobic microorganisms contributed more significantly to estrogen removal.

Comparing the three estrogens, removal efficiencies were in the order of E₂ > E₁ > EE₂, which were related to the adsorption and biodegradation characteristics of each estrogen. Adsorption capacity was determined by the partition coefficient (Kₚ), and logKₚ values for E₁, E₂ and EE₂ were reported in the range of 1.99–2.95, 2.67–2.78 and 2.00–3.35, respectively (Koh et al. 2009), which were between 2 and 4, indicating that adsorption was relevant as a removal mechanism and must rely on degradation studies involving biomass. Ren et al. (2007) also reported that the order of decreasing adsorption affinity onto sludge followed EE₂ > E₁ > E₂. Molecular structure determined estrogen bioavailability. The biodegradation characteristics of the three estrogens have been widely studied. Li et al. (2008) found that an apparent first-order disappearance rate of E₂ and E₁ was in the range of 0.84–4.51 and 0.15–0.84 h⁻¹, while the biodegradation half-lives of EE₂ were reported from 7 h to several days. These data suggested that biodegradation was more crucial for E₂ removal, although its adsorption ability was weakest in the three estrogens.

**Effect of SRT on the removal of three estrogens**

The overall estrogen removal efficiencies and percentage removal by each unit are shown in Figure 2(b). When SRT was increased from 10 to 20 d, the overall removal efficiencies of E₁ first increased from 80.0 to 91.7%, then decreased to 86.6%, with the maximum value at an SRT of 15 d; Those of E₂ all increased from 81.8 to 98.8%, with the largest value at an SRT of 20 d; those of EE₂ were directly reduced from 87.2 to 80.9%, with the best SRT of 10 d. Taken into account the removal of all three estrogens, an SRT of 15 d was much more suitable. Too short an SRT meant that microorganisms with the ability to biodegrade estrogen could not acclimate and develop in the system, leading to low removal of estrogen. An increase of SRT could allow the development of specific organisms of slow growth (such as nitrifying bacteria, ammonia-oxidizing bacteria, etc.) and establishment of a more diverse biocoenosis within biological reactors, which has been proved with high physiological capabilities and greater potential for estrogen degradation in wastewater (Shi et al. 2004). However, too long an SRT might reduce estrogen removal efficiencies by increasing the organic fraction inert in the more stabilized biomass. The optimal removal conditions for estrogen were also relevant to bioreactor structure and estrogen species. Ifelebuegu (2011) reported that removal efficiencies for estrogens in treatment processes were in the order of activated sludge > oxidation ditch > biofilter > rotating biological contactors. Estrada & Mijaylova (2011) found removals higher than 99.9% for all estrogens with an SRT of 60 days and an HRT of 12 h in the membrane bioreactor.

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**Figure 2** | Removal efficiencies of the three estrogens in the overall system and each biological tank at different HRTs (a) and SRTs (b).
Anaerobic, anoxic and aerobic reactors respectively contributed about 11.5–18.8%, 12.4–18.7% and 24.8–48.1% to total E1 removal, 6.4–27.5%, 10.7–10.8% and 57.3–71.6% to total E2 removal, and 9.1–33.7%, 6.3–10.9% and 20.3–60.6% to total EE2 removal, which indicated that aerobic microorganisms were significant for estrogen removal. These data were consistent with the data in the HRT variation experiment. However, further research is needed to determine other factors that enhance estrogen removal, as well as to investigate possible metabolites formed in biological treatment.

The optimal HRT and SRT for both estrogens and nutrients

Owing to the fact that the AAO system is usually designed for nutrient removal, conventional parameters (such as COD, NH₄⁺-N, TN, TP and TOC) were also measured to determine the optimal HRT and SRT for both nutrient and estrogen removal. As shown in Figure 3, the removal efficiencies of TN and TOC increased, respectively, from 65.2 and 69.9% to 86 and 92.8% with HRT increasing from 5 to 15 h; COD, NH₄⁺-N and TP reached the highest rate at an HRT of 8 h with the values of 93.9%, 89.9% and 77.5%, respectively. Therefore, the most suitable HRT for both nutrient and estrogen removal seemed to be 8 h.

As shown in Figure 3, changing SRT from 10 to 20 d had no significant effect on COD, while increasing the removal efficiencies of NH₄⁺-N, TN and TOC. Total phosphorus had the highest removal rate at an SRT of 15 d, and reduced evidently at an SRT of 20 d. This was because biological phosphorus removal mainly relied on the excess sludge, which was produced in large amounts at a shorter SRT. There was little difference in COD, NH₄⁺-N and TN removal between SRTs of 15 and 20 d. Therefore, 15 d might be the optimal SRT for both nutrient and estrogen removal.

The fate and mass flux of three estrogens in the AAO process

The fate of estrogens in the AAO system included accumulating in the system, discharging by final effluent and waste sludge, and being degraded by activated sludge. The mass balance of estrogens in the AAO system can be expressed by Equation (1):

\[ M_{\text{influent}} = M_{\text{accumulation}} + M_{\text{effluent}} + M_{\text{excess-sludge}} + M_{\text{biodegradation}} \]

where \( M_{\text{influent}}, M_{\text{effluent}} \) and \( M_{\text{excess-sludge}} \) were estrogen mass flow in influent, final effluent, and excess sludge (μg/d), which can be calculated based on estrogen concentrations. \( M_{\text{accumulation}} \) was estrogen mass flow accumulated in the AAO system (μg/d), which can be obtained from sludge mass and estrogen concentrations in the anaerobic, anoxic, oxic and settling tanks. \( M_{\text{biodegradation}} \) was the estrogen mass flow degraded by microorganisms in the system (μg/d), which was calculated by Equation (1).

Under optimal conditions for nutrient and estrogen removal (HRT = 8 h and SRT = 15 d), the distribution law and fate of the three estrogens in each tank were investigated by detecting estrogen concentration in the aqueous and solid phase. As shown in Table 1, E₁, E₂ and EE₂ concentrations in the influent were about 15 μg/L, while their effluent concentrations were only 1.33, 0.84 and 2.74 μg/L, with overall removal efficiencies of 89.97, 94.60 and 82.57%, respectively, which was related to the structure and characteristics of the estrogens. The E₁, E₂ and EE₂ quantities in the sludge tank were 30.14–42.38 μg/g, 18.57–25.36 μg/g and 64.15–78.41 μg/g, respectively. These data suggested that E₂ had lowest adsorption content on sludge, which was determined by the low logKₐw (3.1–4.0) and the rapid degradation rate in the reactor. By comparison, EE₂ with the characteristic
of a higher logKOW (4.15) and, being resistant to biodegradation, was easily adsorbed to the sludge. Furthermore, the three estrogens had different adsorbed quantities on anaerobic, anoxic, oxic and returning sludge. For example, adsorbed EE2 on sludge had the order of anoxic sludge > anaerobic sludge > returning sludge > oxic sludge, which was consistent with our previous study (Zhang et al. 2012). The E1 and E2 had the largest adsorbed content on returning sludge and anoxic sludge, which was related to the character, shape, particle size and organic content, etc.

The mass flow and mass balance of three estrogens were determined to assess their potential removal mechanisms in the AAO reactor (Figure 4). In the influent, mass flows of the three estrogens were about 483, 471 and 475 μg/d for E1, E2 and EE2, which were reduced by 54.6, 80.9 and 54.0%, respectively, after biological treatment units. It indicated that biological degradation was crucial to estrogen removal. Low mass change percentages and variability in anaerobic and anoxic treatment units suggested that biodegradation in these two units was of minor importance in estrogen removal. By comparison, removal of E1, E2 and EE2 in the aerobic tank were 50.1, 68.5 and 27.6%, respectively, suggesting that biodegradation was much more significant in this unit. For secondary effluents, mass flows of the three estrogens were 40, 25 and 82 μg/d, with removal efficiencies of 82.1, 72.6 and 63.9%, respectively, in the secondary tank, which indicated that estrogens were apt to adsorb on sludge in the precipitation process. The estrogen mass flow in excess sludge was 59, 34 and 97 μg/d, accounting for 12.2, 7.2 and 20.4% of influent mass flows, respectively, which suggested that excess sludge was a sink of estrogen in wastewater treatment and had a potential implication for the environment.

Based on estrogen data at an HRT of 8 h and an SRT of 15 d, as shown in Table 1, the fate of influent estrogens is presented in Figure 5. For E1, 50.7% was due to degradation by activated sludge, 28.8% was accumulated in the system, 8.3% was discharged in effluent, and 12.2% remained in waste sludge, which indicated that biodegradation and adsorption of activated sludge were the main removal mechanisms of E1; for E2, 70.1% of influent was biodegraded by microbes, 17.2% was accumulated in the reactor, 5.4% remained in effluent, and 7.3% was in excess sludge, which showed that biodegradation was the most important removal mechanism of E2; for EE2, only 11.3% was degraded by microorganisms, more than 50% was accumulated in the system, 17.3% was discharged in effluent, and

<table>
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<tr>
<th>Compounds</th>
<th>E1 (μg/L)</th>
<th>E2 (μg/L)</th>
<th>EE2 (μg/L)</th>
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</thead>
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<td>Influent</td>
<td>15.96</td>
<td>15.57</td>
<td>15.72</td>
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<tr>
<td>Anaerobic Tank</td>
<td>Water (μg/L)</td>
<td>7.86</td>
<td>7.91</td>
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<td></td>
<td>Sludge (μg/g)</td>
<td>32.56</td>
<td>21.12</td>
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<tr>
<td>Anoxic Tank</td>
<td>Water (μg/L)</td>
<td>5.26</td>
<td>4.78</td>
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<td></td>
<td>Sludge (μg/g)</td>
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<td>25.36</td>
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<td>Aerobic Tank</td>
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<td>1.50</td>
</tr>
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<td></td>
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<td>37.93</td>
<td>18.57</td>
</tr>
<tr>
<td>Effluent</td>
<td>Water (μg/L)</td>
<td>1.33</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>Sludge (μg/g)</td>
<td>42.38</td>
<td>24.74</td>
</tr>
</tbody>
</table>

Figure 4 | Mass flux of the three estrogens in the AAO process system: (a) anaerobic tank, (b) anoxic tank, (c) aerobic tank, (d) secondary clarifier, (e) excess sludge.
20.34% was transported into excess sludge, which suggested that sludge adsorption was the main removal mechanism of EE2.

CONCLUSIONS

The selection of operating parameters in the AAO process was crucial to estrogen removal in wastewater. When HRT was increased from 5 to 15 h, removal of all three estrogens showed the trend of first increasing and then decreasing, with the highest values of 91.2, 94.6 and 81.5% for E1, E2 and EE2, respectively. In an SRT range of 10–20 d, the optimal SRT for the removal of the three estrogens was different, with removal efficiencies all above 80%. The optimal HRT and SRT for the removal of both nutrients and the three estrogens should be 8 h and 15 d, respectively. Under these conditions, the percentage of four fates (biodegradation by sludge, accumulation in system, discharging in effluent and remaining in excess sludge) were 50.7, 28.8, 8.3 and 12.2% for E1, 70.1, 17.2, 5.4 and 7.3% for E2, and 11.3, 50, 17.3 and 20.34% for EE2, respectively. The main removal mechanisms for E1 and E2 were biodegradation by activated sludge, while for EE2 removal, it was adsorption on sludge.

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