Fate of 17β-estradiol and 17α-ethinylestradiol in batch and column studies simulating managed aquifer recharge

Sung Kyu Maeng, Saroj K. Sharma, Jae Woo Lee and Gary L. Amy

ABSTRACT

Laboratory-scale batch and soil columns experiments were conducted to investigate the attenuation of estrogens (17β-estradiol and 17α-ethinylestradiol) during managed aquifer recharge. The role of microbial activity in the removal of selected estrogens was evaluated by comparing the results from biotic and abiotic batch experiments. Moreover, batch experiments were carried out using the sand media prepared over different acclimation periods to investigate the impact of acclimation periods on the removal of selected estrogens. Batch studies showed that adsorption was the dominant removal mechanism in the removal of 17β-estradiol and 17α-ethinylestradiol. 17β-estradiol and 17α-ethinylestradiol were attenuated by 99% and 96%, respectively, in batch experiments under oxic conditions. Redox conditions did not show any significant effect on the attenuation of 17β-estradiol. However, the net estrogenicity of 17β-estradiol remaining was lower under oxic conditions (130 ng estradiol-equivalents/L) than anoxic conditions (970 ng estradiol-equivalents/L). Column studies operated at 17 h of empty bed contact time also demonstrated that removal mechanism of 17α-ethinylestradiol was more dependent on adsorption than biodegradation.

Key words | 17α-ethinylestradiol, 17β-estradiol, estrogens, managed aquifer recharge, microbial activity, redox conditions

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INTRODUCTION

A number of studies have reported the feminization of male aquatic species in receiving waters significantly influenced by the effluents of wastewater treatment plants and surface runoff from agricultural activities and municipal biosolids (Khanal *et al.* 2006). Wastewater treatment plants receive a large variety of endocrine disrupting compounds (EDCs) but are not always effective in removing these compounds with conventional wastewater treatment systems before discharging effluent into receiving water bodies. Previous studies have reported that the occurrence of estrogens was diverse in surface waters, wastewater, and treated wastewater (Carballa *et al.* 2004; Cargouët *et al.* 2004; Nakada *et al.* 2005; Hintemann *et al.* 2006; Lishman *et al.* 2006; Sarmah *et al.* 2006; Ma *et al.* 2007). In addition, this is a concern for water utilities where their raw water is strongly influenced by wastewater effluents. Therefore, there is a potential for the presence of residual EDCs such as estrogens in drinking water sources that could adversely impact human health. Moreover, little attention has been paid to the estrogenicity of transformation products that remain in drinking water resources.

Among the variety of EDCs, 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) have shown the most estrogenicity, while alkylphenols and their ethoxylates have shown less estrogenicity than that of estrogens (Gomes & Lester 2003). Moreover, E2 and EE2 possess estrogenic potency 10,000 to 100,000 times higher than exogenous EDCs such as organochlorine aromatic compounds (Gomes & Lester 2003; Hanselman *et al.* 2003; Cargouët *et al.* 2004). Natural estrogens (E2) and synthetic estrogens (EE2) have received the most scientific attention and are classified as EDCs. Ternes *et al.* (1999) showed that E2 was frequently present in effluents discharged from German and Canadian

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sewage treatment plants at a range of ng/L. However, further studies are needed to determine the effects of EDC concentrations detected in wastewater effluents on aquatic environment and human health.

Managed aquifer recharge (MAR) systems, including riverbank filtration, lake bank filtration, and artificial recharge and recovery are commonly used for the intake of drinking water sources in European countries (Maeng *et al*. 2011). MAR systems are natural, robust, and cost-effective treatment or pre-treatment technologies for reducing bulk organic matter, pathogens, and contaminants of emerging concern such as estrogens, allowing for the supply of safe drinking water (Heberer & Adam 2004; Grünheid & Jekel 2005; Mechlinski & Heberer 2005; Massmann *et al*. 2008; Maeng *et al*. 2008, 2012a, b). E2 and EE2 have high octanol-water partition coefficients (K_{ow}) indicating that these compounds are expected to easily adsorb onto soil due to their hydrophobic properties (Table 1).

Layton *et al*. (2000) used the ^{14}C-labeled E2 compound to demonstrate its fate during the activated sludge process (i.e., conventional wastewater treatment process), and the process was found to be capable of mineralizing 70 to 80% of E2 to carbon dioxide in 24 h. Therefore, E2 could be removed through biodegradation. However, no previous studies have compared the removal of E2 and EE2 between adsorption and biodegradation during soil passage. Moreover, proper understanding and analysis of the fate of E2 and EE2 during soil passage is essential to optimize their removal in MAR systems. The removal of transformation products should also be considered beyond the attenuation of the parent compounds because the transformation products could still possess estrogenic activity. Therefore, total estrogenic activity remaining either in transformation products or parent compounds must be also considered in order to accurately estimate the overall elimination of EDCs. However, previous studies have not addressed the estrogenic activity remaining during MAR systems.

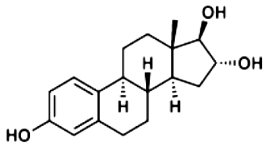
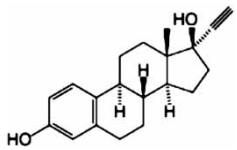
The main goal of this study was to evaluate the fate of E2 and EE2 in batch experiment simulating MAR. Specific objectives to achieve this goal were: (1) to determine the relative performance of biodegradation and adsorption for E2 and EE2, (2) to investigate the fate of E2 and EE2 under different acclimation periods, and (3) to determine the remaining estrogenic activity from E2 using an estrogen-responsive chemically activated luciferase expression (ER-CALUX) assay.

MATERIALS AND METHODS

Chemicals

Standards of E2 and EE2 were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Individual stock solutions

Table 1 | Physiochemical properties of steroid estrogens

	17β-Estradiol	17α-Ethinylestradiol
Structure		
Formula	$C_{18}H_{24}O_2$	$C_{20}H_{24}O_2$
Vapor pressure (mmHg)	2.3×10^{-10}	4.5×10^{-11}
Solubility in water at 20 °C (mg/L)	13	4.8
Octanol-water partitioning coefficients (log K_{ow})	3.94	4.15
Molecular weight g/mol	272.4	296.4
Natural estrogen	Yes	No
Acronyms	E2	EE2

Source: Modified and reproduced from Ying *et al*. (2002).

of E2 and EE2 were prepared in methanol (100 mg/L), and an additional carbon source from methanol was minimized as low as possible when selected estrogens were introduced to feed waters. Physicochemical properties of E2 and EE2 are shown in Table 1.

Dissolved organic carbon (DOC) and adenosine triphosphate (ATP)

Organic matter in all samples was characterized within 3 d after sample collection and storage at 4 °C after 0.45 μm filtration to prevent biodegradation of organic matter. The concentration of bulk organic matter was determined as DOC by a total organic carbon analyzer (Shimadzu TOC-VCPN, Japan). The active biomass associated with sand in batch and column studies was determined by ATP. Wet sand samples between 2 and 4 g collected from batch reactors and soil columns with 50 mL of autoclaved tap water were sonicated at a power of 40 W in order to obtain the biomass (Branson W-250D Sonifier; microtip diameter, 5 mm). A detailed description of methods used for the ATP measurements is explained in Magic-Knezev & vsn der Kooij (2004).

E2 and EE2 analyses

E2 and EE2 were determined using enzyme-linked immunosorbent assay (ELISA; Japan EnviroChemicals Ltd). Previous studies have shown that good correlations were achieved between ELISA and conventional analytical analysis (high-performance liquid chromatography, liquid chromatography–tandem mass spectrometry (LC-MS/MS), and gas chromatography–mass spectrometry) in the measurement of estrogens, and ELISA has been used to investigate the fate of estrogens in environmental samples (e.g., river sediment, manure, wastewater, activated sludge, industrial wastes, etc.) (Farré *et al.* 2006; Hirobe *et al.* 2006; Vigano *et al.* 2008; Zhao *et al.* 2009; Ho *et al.* 2011). ELISA is based on a competitive reaction where enzymes labeled standard E2 and EE2 compete with free E2 and EE2 in a sample for binding to a specific monoclonal antibody immobilized to the surface of a microplate. The amount of labeled E2 and EE2 bound to the antibody is determined using a microplate reader (Anthos Labtec HTIII, Salzburg, Austria). The color intensity is measured at a wavelength of 450 nm and allows the quantification of E2 and EE2 concentration. All samples were extracted by solid phase extraction (SPE) following the method reported by Farré *et al.* (2006). SPE cartridges (Oasis HLB C18 cartridge 6 cc, 1 g) were obtained from Waters. In our study, recovery tests of ELISA kits (E2 and EE2) were conducted in autoclaved tap water (DOC, 2 mg/L) by spiking each E2 and EE2 at 100 μg/L, and average recoveries of E2 and EE2 were 87 and 94%, respectively. All experiments in this study were conducted separately for E2 and EE2 to avoid partial interference that may occur between two selected estrogens during ELISA. The interference between estrogens in ELISA has been reported by comparing results obtained from LC-MS/MS (Farré *et al.* 2006).

Estrogenic activity

A commercially available immunoassay, ER-CALUX assay was used to provide information on the estrogenic potential of environmental samples, which include transformation products originating from parent compounds and carried out by BioDetection Systems b.v. (Amsterdam, The Netherlands). The luciferase luminesces (i.e., an amount of luciferase production) was quantitatively measured using a luminometer. The results are expressed as ng estradiol-equivalent/L of water. More information on the ER-CALUX protocol is described elsewhere (Legler *et al.* 1999, 2002).

Batch and column setup

Batch reactors were set up with 100 g of standard silica sand between 0.8 and 1.25 mm in eight batch reactors (duplicate) filled with Delft canal water (DCW) (Delft, The Netherlands) and secondary effluent (SE) (1:1) up to 400 mL. The SE was collected from a wastewater treatment plant located in Hoek van Holland, The Netherlands. A 60 d acclimation period was necessary to stabilize the batch reactors with respect to DOC removal (fill-and-draw mode during the acclimation period, hydraulic retention time (HRT) 5 d). Batch experiments under biotic conditions were conducted to investigate the removal of E2 and EE2 during MAR. Sodium azide (NaN_3) was used to inhibit microbial activity, and the concentrations of sodium azide required for inactivating the activity of microorganisms

associated with sand (establishing abiotic conditions) were also pre-determined using DCW and SE (1:1). Three different concentrations (2, 10, and 20 mM) of sodium azide were tested in order to assess abiotic conditions, and ATP was measured to determine the microbial activity associated with sand.

The fate of EE2 during soil passage was investigated by conducting laboratory-scale soil column studies. Three columns (SC1, SC2, and SC3) were deployed, and the experiments were carried out in a dark room. The soil columns were 300 mm long and comprised of inner and outer diameters (50 mm inner diameter, XK50/30, Amersham Pharmacia Biotech, Sweden). Silica sand between 0.8 and 1.25 mm was used as the granular media, and the hydraulic loading rate was maintained at 0.64 m/d. The inner part was packed with silica sand, while the outer one was used to maintain operating temperatures between 16 and 17 °C by flowing water from a chiller. SC1 received tap water (DOC of 2 mg/L) from non-chlorinated distribution systems (Delft, The Netherlands). SC2 and SC3 were conducted under abiotic and biotic conditions, respectively. 20 mM of sodium azide was enough to suppress the microbial activity associated with sand in SC2. The concentration of sodium azide was determined from an abiotic control experiment. Columns were acclimated for 60 d using SE and DCW (1:1), and empty bed contact time (EBCT) was 17 h.

RESULTS AND DISCUSSION

Abiotic batch experiments using sodium azide

Abiotic experiments using batch reactors under oxic conditions were necessary to determine whether it is feasible to use sodium azide as a biocide to suppress aerobic microbial activity. After a 60 d acclimation period, sodium azide was added to batch reactors at 0, 2, 10, or 20 mM. DOC removals after 5 d are presented in Figure 1. A DOC reduction of 24% was achieved in the control reactor (sodium azide at 0 mM). However, the DOC removals were lowered below 10% when sodium azide was added at 10 or 20 mM, which demonstrated that more than 50% of the DOC removal was attributed to biodegradation (Figure 1). Furthermore, batch reactors fed with sodium

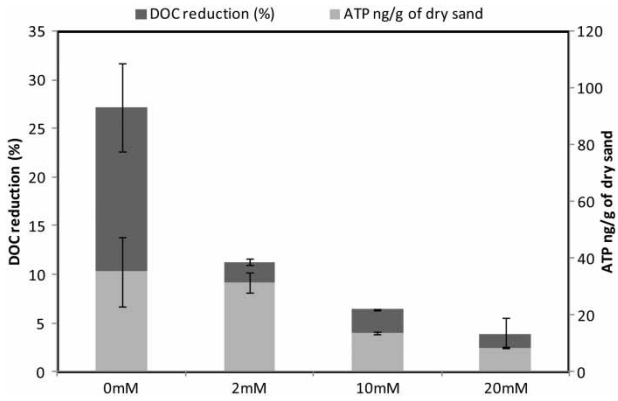


Figure 1 | Dissolved organic carbon (DOC) removal in batch reactors with different concentrations of sodium azide (HRT = 5 d, $n = 3$).

azide exhibited low ATP, indicating that sodium azide had suppressed the microbial activity associated with sand. In this study, a sodium azide concentration greater than 10 mM was necessary to suppress microbial activity associated with sand in terms of ATP. It is important to note that the concentration of sodium azide must be determined prior to conducting the abiotic experiments, and the concentration may vary under different conditions (e.g., total active biomass concentration, soil organic matter, etc.). Higher concentrations of sodium azide may be necessary to suppress the microbial activity that is encapsulated with soil organic matter.

Adsorption isotherms of E2 and EE2

The distribution coefficients (K_d) for E2 and EE2 were determined using the slope of the linear adsorption isotherms. At the start, E2 and EE2 standards were added to reactors containing DCW and SE (1:1) with 40 g of autoclaved silica sand (dry weight) at initial E2 and EE2 concentrations of 20, 40, 80, and 100 μg/L. All of the reactors were maintained at 20 °C and were separately prepared to eliminate any possible interference between E2 and EE2 during ELISA analyses. The K_d values for E2 and EE2 were 169 and 65 mL/g. The adsorption of E2 and EE2 by sand increased linearly with increasing E2 and EE2 concentration. A previous study also reported the distribution coefficients, K_d (mL/g), of E2 and EE2 in sand, in a sandy loam and in a silt loam soil under abiotic conditions (Karnjanapiboonwong *et al.* 2010). This study determined that K_d values of

E2 were 3.8, 115.8, and 198 mL/g in sand, in the sandy loam and in the silt loam soil, respectively, and K_d values of EE2 were 1, 176.2, and 196.8 mL/g, respectively. The K_d values of E2 and EE2 obtained in this study were within the range of the previous study.

Influences of microbial activity and acclimation period on the attenuation of E2 and EE2

Batch reactors were deployed to investigate microbial activity associated with sand on the removal of E2 and EE2 during MAR. Adsorption was the dominant mechanism in the attenuation of E2 and EE2 during MAR, and an additional attenuation could be achieved due to microbial activity (Mansell & Drewes 2004). There are several environmental conditions that may influence the removal mechanisms (adsorption and/or biodegradation) of E2 and EE2 during soil passage. These factors include microbial activity associated with sand and acclimation periods. During their operation, the overall performance of MAR systems can vary based on factors simulated under different environmental conditions.

Based on the abiotic experiment above, a sodium azide concentration of 20 mM significantly decreased the microbial activity associated with sand. However, E2 and EE2 removals were not significantly changed under different microbial activities (Figure 2). Mansell & Drewes (2004) observed that attenuation of E2 and EE2 increased in the presence of microbial activity, but they determined that the majority of attenuation of E2 and EE2 was due to adsorption. It appears that adsorption was the main removal mechanism for E2 and EE2 due to their hydrophobicity (high log K_{ow}). These findings confirm that the hydrophobicity of a compound plays an important role in the removal of E2 and EE2 during MAR. Non-chlorinated water was also used to suppress the microbial activity by supplying low biodegradable organic matter. Tap water in the Netherlands is biostable with an assimilable organic carbon (AOC) of about 10 μg/L. The ATP levels associated with sands in E2 and EE2 experiments were reduced with non-chlorinated water, resulting in the lowering of microbial activity associated with sand (Figure 3), yet more than 90% of E2 and EE2 were still attenuated. Therefore, this study reveals that supplying low biodegradable organic matter such as non-chlorinated water can reduce microbial activity.

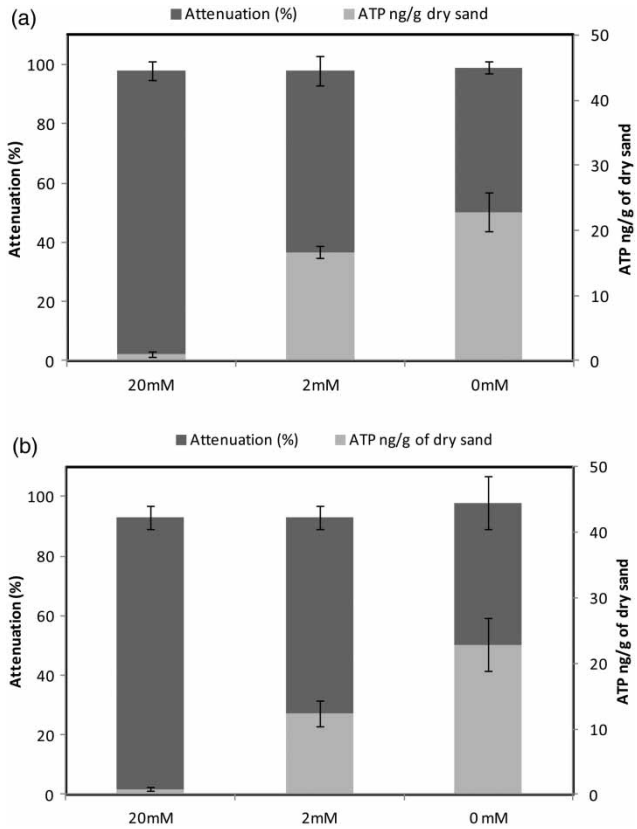


Figure 2 | Attenuation of E2 (a) and EE2 (b) under different microbial activity associated with sand (sodium azide at 0, 2, and 20 mM, $n = 3$).

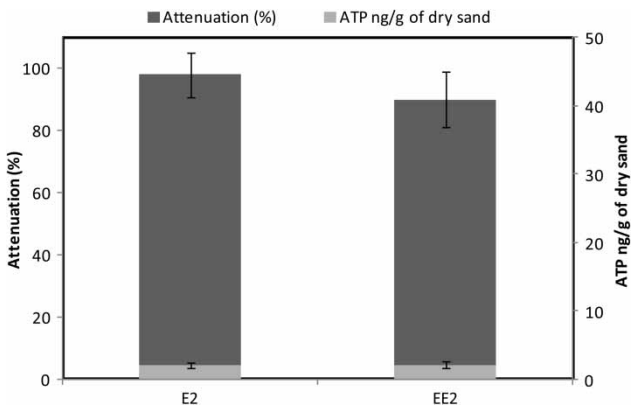


Figure 3 | Attenuation of E2 and EE2 in batch reactors fed with non-chlorinated tap water to suppress microbial activity.

Acclimation periods are important in MAR systems because the top layer of riverbed sediment (bioactive sand) may be disturbed during flooding periods. In this study, autoclaved sands and 60 d acclimated sands were compared to determine whether acclimation periods could

affect the removal of E2 and EE2. Microbial activities in batch reactor without acclimation were significantly lower compared to batch reactors acclimated for 60 d. However, acclimation periods did not affect the removal of E2 and EE2 (Figure 4).

Estrogenic activity under different redox conditions

In general, redox conditions from oxic to anoxic conditions are often observed during soil passage, but this is dependent upon source water quality and type of MAR systems (e.g., bank filtration – prolonged anoxic conditions versus artificial recharge recovery – more oxic conditions) (Maeng *et al.* 2011). Previous studies demonstrated that E2 was effectively removed in aquifer material-water mixtures under different redox conditions (Ying *et al.* 2003; Mansell *et al.* 2004). Therefore, different redox conditions in MAR systems are not likely to affect the removal of E2. However, the estrogenic activity needs to be determined in order to estimate their complete removals. Therefore, in this study, estrogenic activity determined by ER-CALUX was assessed under different redox (biotic) conditions to investigate the estrogenic activity remaining from E2 which has been shown to transform to estrone (E1) in river water with half-lives of 0.2 to 9 d at 20 °C (Jürgens *et al.* 2002). Moreover, it is considered an incomplete elimination of estrogenic activity because E1 still consists of some estrogenicity level (0.1–0.2 of E2 equivalent) (Nghiem *et al.* 2004). Therefore, it is important to note that estrogenic intermediates (i.e., transformation products) from a parent compound (E2) should be carefully monitored. In our study, estrogenic activity remaining in batch reactors was significantly attenuated. The estrogenic activity remaining in filtrate under oxic conditions (130 ng estradiol-equivalents/L) was relatively low compared to that under anoxic conditions (970 ng estradiol-equivalents/L).

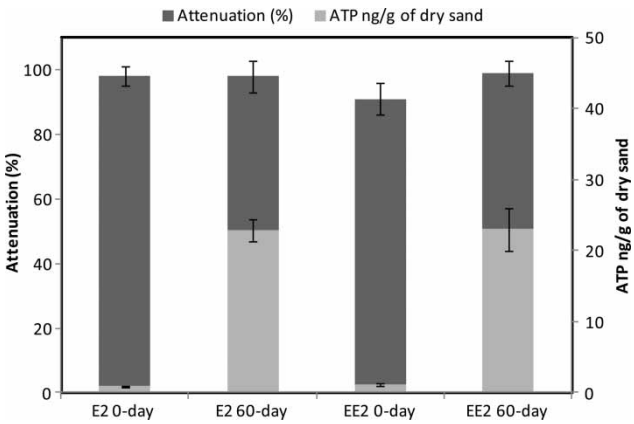


Figure 4 | Attenuation of E2 and EE2 in batch reactors prepared in different acclimated periods.

Soil column study

Soil column studies were also carried out to assess the fate of EE2 during soil passage. The average removal efficiencies of DOC for SC1, SC2, and SC3 were about 12%, and EE2 was introduced after the acclimation periods (60 d). After the acclimation periods, SC1 was fed with non-chlorinated water (a low carbon source), and DCW and SE (1:1) were introduced into SC2 and SC3, respectively. SC2 was maintained under abiotic conditions using sodium azide, and SC3 remained under biotic conditions. EE2 removals for SC1, SC2, and SC3 were 64, 67, and 87%, respectively. It can be argued that the removals in SC1 and SC2 are mainly due to adsorption, and the removal in SC3 is due to the combination of adsorption and biodegradation. Adsorption appeared to be the major removal mechanism for EE2 removal; however, the difference between SC2 and SC3 indicated that biodegradation also contributed to the reduction of EE2. In a previous study, EE2 was found to be degraded in aquifer material acclimated with effluent (Ying *et al.* 2008). Therefore, it is also necessary to consider biodegradation as a removal mechanism when estimating the fate of EE2 over short contact time (EBCT: 17 h) during MAR. It is hypothesized that EDCs first adsorb onto the media and then slowly undergo biodegradation over time. Therefore, the adsorption capacity may be regenerated and not exhausted with time. Moreover, the EE2 removal was between 64 and 87% at a depth of only 300 mm, but a higher removal was expected in a MAR system in which travel distances are generally greater than 10 m.

CONCLUSIONS

Based on the experimental results, the following conclusions can be drawn:

- The abiotic experiment using sodium azide suppressed the microbial activity associated with sand. However, it is important to note that an abiotic experiment for MAR needs careful consideration in inactivating micro-organisms encapsulated in soil organic matter. Therefore, it is necessary to measure the microbial activity associated with the soil organic matter prior to conducting the abiotic experiment.
- Batch experiments showed a significant removal of E2 and EE2, and estrogenic activity measured by ER-CALUX indicated that most of the remaining estrogenicity (ng estradiol-equivalents/L) in filtrates was eliminated (HRT: 5 d under oxic conditions).
- The estrogenic activity remaining in filtrate under oxic conditions (130 ng estradiol-equivalents/L) was relatively low compared to that under anoxic conditions (970 ng estradiol-equivalents/L).
- Soil column studies showed that EE2 removals for SC1, SC2, and SC3 were 64, 67 and 87%, respectively. These column studies confirmed that adsorption appeared to be the major removal mechanism for EE2; however, the difference of EE2 removal between SC2 (abiotic) and SC3 (biotic) revealed that biodegradation also slightly contributed to the reduction of EE2 over short EBCT (17 h).

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