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Microalgae cultivation on wastewater digestate: β -estradiol and 17 α -ethynylestradiol degradation and transformation products identification

Andrea Hom-Diaz^a, Marta Llorca^b, Sara Rodríguez-Mozaz^b, Teresa Vicent^a, Damià Barceló^{b,c}, Paqui Blánquez^{a,*}

^a Chemical Engineering Department, Escola d'Enginyeria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

^b Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, C/Emili Grahit, 101-E17003 Girona, Spain

^c Water and Soil Quality Research Group, Department of Environmental Chemistry (IDAEA-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

*Corresponding author: Paqui Blánquez

Chemical Engineering Department

Escola d'Enginyeria

Universitat Autònoma de Barcelona

08193 Cerdanyola del Vallès. Spain

E-mail address: paqui.blanquez@uab.cat

Tel: +34 935811879; Fax: +34 935812013

Abstract

Selenastrum capricornutum and *Chlamydomonas reinhardtii* were tested for possible biodegradation of the hormones β -estradiol (E2) and 17 α -ethinylestradiol (EE2) when cultured in anaerobic digester centrate (ADC). Neither ADC nor the hormones had a negative or toxic effect on the microalgae growth but enhanced it. E2 and EE2 biodegradation was evaluated under different culture conditions. After 7 days of treatment, between 88% and 100% of E2 was removed by *S.capricornutum*. Overall, 42 and 54% of the removal was attributed to biodegradation processes, while the rest of the removal was due to adsorption onto the algae biomass. For EE2, removals between 60 and 95%, depending on the culture conditions, were achieved, with biodegradation accounting for 20-54% of the removal. E2 and EE2 were completely removed in the experiments performed with *C.reinhardtii*, except for EE2 in the presence of ADC, which decreased to 76%. However, *C.reinhardtii* presented higher adsorption percentages: 86% and 71% after 7 days for E2 and EE2, respectively. Transformation products (TPs) of E2 and EE2 generated in each treatment were also monitored. Two TPs were tentatively proposed as degradation products of E2 and EE2 by the algae. In addition, the removal of 26 endocrine disruptors and related pollutants present in the centrate was also monitored: bisphenol A was completely removed, whereas tris(2-butoxyethyl)phosphate was only removed in the absence of hormones.

Keywords

Selenastrum capricornutum, *Chlamydomonas reinhardtii*, hormones, transformation products, anaerobic digestion centrate

1 Introduction

Estrogenic activities in wastewater treatment plant (WWTP) effluents have previously been described by several authors and were attributed to the presence of natural estrogens, such as β -estradiol (E2), and synthetic estrogens, such as 17 α -ethynylestradiol (EE2) (Chang et al., 2011; Jarošová et al., 2014). These compounds are excreted in the urine by women and therefore end up in wastewater effluents. They can cause severe damage because of their endocrine disrupting effects, provoking several negative effects on the environment and human and wildlife health, especially in aquatic environments, even at concentrations below 10 ng/L (Rudder et al., 2004). E2 and EE2 are currently under review for identification as priority substances from the last proposal from the European Commission (European Commission, 2007) due to their high estrogenic potency. E2 and EE2 have been described as the most important micropollutants in municipal WWTPs (Rudder et al., 2004). Although some urban WWTPs have been reported to remove up to 98% of E2 (Servos et al., 2005), Snyder et al. (1999) determined that EE2 is not removed. Due to the low biodegradability of these compounds, they are found in the environment (Esteban et al., 2014). The fate of hormones during sludge treatment has been poorly documented. Only a few studies have reported the fate and behavior of estrogenic hormones in anaerobic sludge treatment at lab scale and full scale experiments (Ifelebuegu, 2011; Muller et al., 2010). In these studies, it was found that in full scale experiments, the removal percentages are lower (18-32% for E2 and 10-15% for EE2) and that the removal mechanisms are mainly sorption onto sludge biomass. Another study reported removal efficiencies of approximately 50% in sludge anaerobic digesters (Paterakis et al., 2012). Recently, Hamid and Eskicioglu (2013) assessed the fate of steroidal hormones during sludge anaerobic digestion pre-treated by microwaves and reported removal efficiencies between 50-70%.

Because of the difficulty of removing hormones, there is a need to find a treatment to remove these compounds before they are discharged into surface waters to prevent any toxic effects in the environment. Several studies have been conducted to find an appropriate treatment for the removal of E2 and EE2; some examples of treatments are the use of manganese oxide (Rudder et al., 2004), sorption of EE2 on activated charcoal (Kumar and Mohan, 2011), and advanced techniques such as photo-catalysis (Ohko et al., 2002). Biological studies have employed fungi, such as *Trametes versicolor* and *Ganoderma lucidum* (Blázquez and Guieysse, 2008), or bacteria, such as *Sphingobacterium* sp. JCR5 (Haiyan et al., 2007). Microalgae can be used as low-cost systems for pollutant elimination treatments, through photooxidation and/or biodegradation. Della Greca et al. (2008) studied the removal and biotransformation of EE2 in a synthetic medium using the microalgae *Selenastrum capricornutum* and found that EE2 is transformed into 3 products. The conversion yield was enhanced as cell density increased. In addition, Abreu et al. (2012) and Singh et al. (2011) have reported that algae are able to grow in wastewater and exhibit high nutrient removal efficiencies.

In the present work, a microalgae cultivation study was developed using two microalgae strains, *Selenastrum capricornutum* (formerly known as *Pseudokirchneriella subcapitata* and *Rhapidocelis subcapitata*) and *Chlamydomonas reinhardtii*, to evaluate their potential to biodegrade two estrogens growing on centrate from sludge anaerobic digestion (ADC). Due to the nitrogen and phosphorous content, several wastewater (urine, dairy, urban and digestate) can serve as a major nutrient source for microalgae cultivation (Abreu et al., 2012; Li et al., 2011a; Singh et al., 2011). *S. capricornutum* was selected as model specie because of its ubiquitous occurrence and easy cultivation. *C. reinhardtii* has been previously tested to be grown on wastewater centrate (Li et al., 2011)

2 Materials and methods

2.1 Microalgae strains and cultivation conditions

C.reinhardtii (UTEX ID 2243) and *S.capricornutum* (UTEX ID 1648) were supplied from the culture collection at the University of Texas (UTEX) in agar tubes.

Experimental cultures were carried out in 100 mL Erlenmeyer flasks under continuous fluorescent lamp irradiation ($172\pm 18 \mu\text{mol}/(\text{m}^2\text{s})$ irradiance level, measured by a light meter (LI.189, LI-COR Quantum/Radiometer/Photometer, USA)) at a controlled temperature ($25\pm 1^\circ\text{C}$) and 130 rpm (orbital shaker Kuhner, LS-X, Switzerland).

Each microalgae strain was cultured in growth medium (GM); *S.capricornutum* was grown on BG-11 Medium (Li et al., 2011b) and *C.reinhardtii* on P49 Medium (UTEX, 2012).

2.2 Wastewater

Anaerobic sludge was collected from an urban WWTP located in ‘El Prat de Llobregat’ (Barcelona, Spain), and the ADC was obtained by centrifuging at 3000 rpm for 15 minutes. The ADC used in the study was characterised (pH 7.86, 202 mg/L total suspended solids (TSS), 153 mg/L N-NH₄⁺ and 1150 mg/L chemical oxygen demand (COD)) and stored at -20°C until the set-up of the experiments.

2.3 Chemicals

E2 and EE2 reagents (>98% purity) were purchased from Sigma-Aldrich (Barcelona, Spain). The rest of the endocrine disruptor chemicals (EDCs) analysed within this work were of high purity level, and detail information about them can be found elsewhere (Gorga et al., 2013) .

HPLC grade acetone, acetonitrile, and water (Lichrosolv) 99% were supplied by Sigma-Aldrich (Barcelona, Spain).

All other chemicals used were of analytical grade and were obtained from Sigma–Aldrich (Barcelona, Spain) and Panreac (Barcelona, Spain).

2.4 Experimental set up

Seven different experimental conditions were established in triplicate, for each strain, to evaluate the growth as well as the estrogen degradation (Table 1) over 10 days. The initial microalgae concentration was 100 mg/L dry weight for both strains. Until the 3rd day, when biomass was at its exponential growth period (data not shown and in agreement with Mitra et al., (2012)), no modifications were made. On the 3rd day, Erlenmeyer flasks with experimental conditions B, C, D, E and G were spiked with E2 and EE2 at an initial concentration of 5 mg/L each hormone. Moreover, in Erlenmeyer flasks with conditions F and G, 10 mL of ADC was added on the same day. More detailed information is presented in section S1 in the Supplementary Material (SM).

Table 1 Different experimental conditions for *S. capricornutum* and *C. reinhardtii* and their respective abbreviations

	Experimental condition	Culture	Hormones	Growth medium
A	Axenic Culture	Algae	-	GM
B	Experimental	Algae	E2 + EE2	GM
C	Killed Control	Killed Algae	E2 + EE2	GM
D	Abiotic 1	-	E2 + EE2	GM
E	Abiotic 2 (light protected)	-	E2 + EE2	GM
F	ADC Culture	Algae	-	GM+ADC

G	Experimental	Algae	E2 + EE2	GM+ADC
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2.5 *Algal biomass concentration*

Algae growth was followed during all of the experiments by measuring the optical density of the algal culture at 400 nm for *S.capricornutum* and at 680 nm for *C.reinhardtii* using a UNICAM 8625 UV/VIS spectrophotometer and converting the optical densities into algae dry weight for each sample. A good correlation between weights and optical densities was obtained for each algae in previous experiments.

2.6 *β -estradiol and 17 α -ethynylestradiol determination*

The determination of E2 and EE2 concentrations was done following the method described elsewhere Blázquez and Guieysse (2008). 1 mL samples were taken from each Erlenmeyer flask and extracted with acetone. The samples were diluted 1/1 (v:v) with the solvent, mixed in a vortex (Zx³, Velp Scientifica, Italy) and left for 15 minutes in the ultrasonication device (Branson 3510, Netherlands) before being mixed again and filtered (Millex-GV, PVDF, 0.22 μ m, Millipore). The filtered samples were analysed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) equipped with an UV detector at 220 nm. The column temperature was 30°C, and a sample volume of 20 μ L was injected from a Dionex autosampler. Chromatographic separation was achieved using a GraceSmart RP-18 column. The mobile phase consisted of ultrapure water (A) and acetonitrile (B); the analysis was performed isocratically (60% A) at 1 mL/min. The retention times for E2 and EE2 were 15 min and 21 min, respectively. The quantification limit for both compounds was 0.125 mg/L.

2.7 Identification of transformation products

For the identification of known transformation products (TPs), the strategy followed was based on the so called non-target analysis of known compounds (Llorca and Rodríguez-Mozaz, 2013). For this reason, an in-house library was built based on the accurate masses of the TPs of E2 and EE2 described in the literature as generated from different water treatments. This library was used during the screening of the samples to tentatively identify any suspected TP.

The samples were analysed using on-line turbulent flow chromatography coupled to a high resolution mass spectrometer Orbitrap. More information about the method is presented in section S2 (SM).

2.8 Identification of endocrine disruptors compounds

26 EDCs and related compounds were analysed using an EQuan on-line sample enrichment system (Thermo Fisher Scientific) with turbulent flow chromatographic columns before normal chromatographic separation. The chromatograph was coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). More detailed information can be found elsewhere (Cruz-Morató et al., 2014).

3 Results and discussion

3.1 Growth results

Time-course profiles of *S.capricornutum* and *C.reinhardtii* cell growth are shown in Fig.1. It is observed that during the first growing period (from 0 to the 3rd day), all experimental conditions grew up at the same rate, achieving a biomass dry weight of 110 ± 3 mg/L for *S.capricornutum* and 266 ± 16 mg/L for *C.reinhardtii* on the 3rd day. On the 3rd day, ADC and/or hormones were

added, and the growth of the different microalgae under the different culture medium conditions was evaluated.

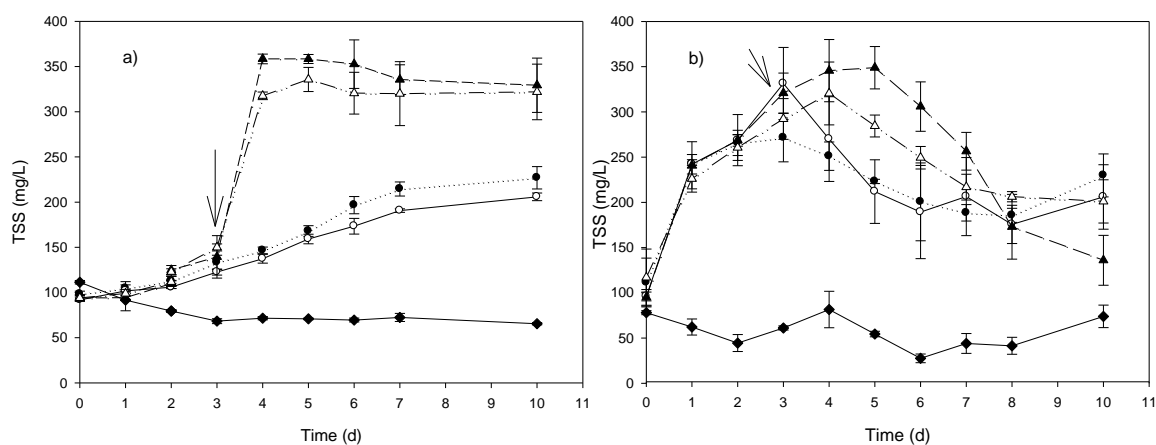


Figure 1 Time-course profile (10 days) of a) *S. capricornutum* growth and b) *C. reinhardtii* growth in terms of dry biomass (A, axenic culture (●); B, algae with hormones (○); C, killed control (◆); F, ADC culture without hormones (▲); G, ADC culture with hormones (△); hormones and/or ADC addition (↓)). Y-error bars represent the standard deviation of three replicates.

The results for *S. capricornutum* showed that growth was enhanced using ADC (conditions F and G), reaching 370 mg/L on the 4th day, whereas without ADC addition (conditions A and B), the biomass increased slightly reaching 150 mg/L. This behavior is consistent with the results reported by several authors who studied the effect of different effluents on microalgae culture growth (Fenton and Ó hUallacháin, 2012; Mitra et al., 2012).

C. reinhardtii did not grow as much as *S. capricornutum* when ADC was added; the biomass on the 4th day was approximately 307 mg/L with ADC (F and G) and 302 mg/L without ADC (A and B). The difference between the strains could be attributed to the fact that the *S. capricornutum* GM contained glucose to establish mixotrophic cell cultivation using light and an organic carbon source. On the contrary, the *C. reinhardtii* medium did not contain carbon;

hence, this strain was less capable of assimilating the carbon coming from the ADC effluent. Abreu et al. (2012) demonstrated that the mixotrophic growth is more efficient for microalgal production, while Moon et al. (2013) stated that *C.reinhardtii* growth under heterotrophic conditions is not beneficial.

From day 4th, the *S.capricornutum* biomass was maintained until the end of the experiment; however, *C.reinhardtii* increased slightly for 2 days after ADC addition and then (from day 5) decreased. As Wang et al. (2012) proposed, successive C-N feeding is the most suitable way to achieve higher cell concentrations and could be evaluated as another means of adding ADC when carbon and nitrogen are depleted.

E2 and EE2 were spiked (conditions B and G) on the 3rd day of the experiment to evaluate whether the estrogens had a negative effect on the algae growth. From Fig.1, comparing A with B and F with G, it can be observed that the growth was not affected when both hormones were added; the tendency is the same. Comparing the results for *S.capricornutum* when estrogens were added (condition B) with the culture (condition A), it can be observed that at the end of the experiment, the growth was only 9% lower in B than in A; however, on the 3rd day, before E2 and EE2 were added, the difference in the biomass concentration was 8% lower. This finding suggests that hormones do not affect strain growth. The same effect was observed under conditions F and G, cultures containing ADC. On the 3rd day, before hormone spiking, the growth in F was 7% lower than that in G, but on the 10th day, the growth was approximately equal. Thus, neither ADC nor hormones inhibited the growth of *C.reinhardtii* or had a toxic effect; the growth decrease is attributed to nutrient depletion.

To disprove the idea of microbial growth being the main consequence of the growth due to the addition of non-sterile ADC, a sample was analysed under a microscope, and primarily algae strains were observed, so microbial growth in ADC was considered minor.

3.2 β -estradiol and 17- α -ethynylestradiol removal

During a 7 day period, after E2 and EE2 were spiked, both concentrations were monitored to determine the ability of each strain to eliminate these compounds. Fig.2 shows the results of E2 and EE2 removal. E2 and EE2 were below the detection limit for the *S.capricornutum* and *C.reinhardtii* controls (A and F), confirming that ADC does not contain detectable concentrations.

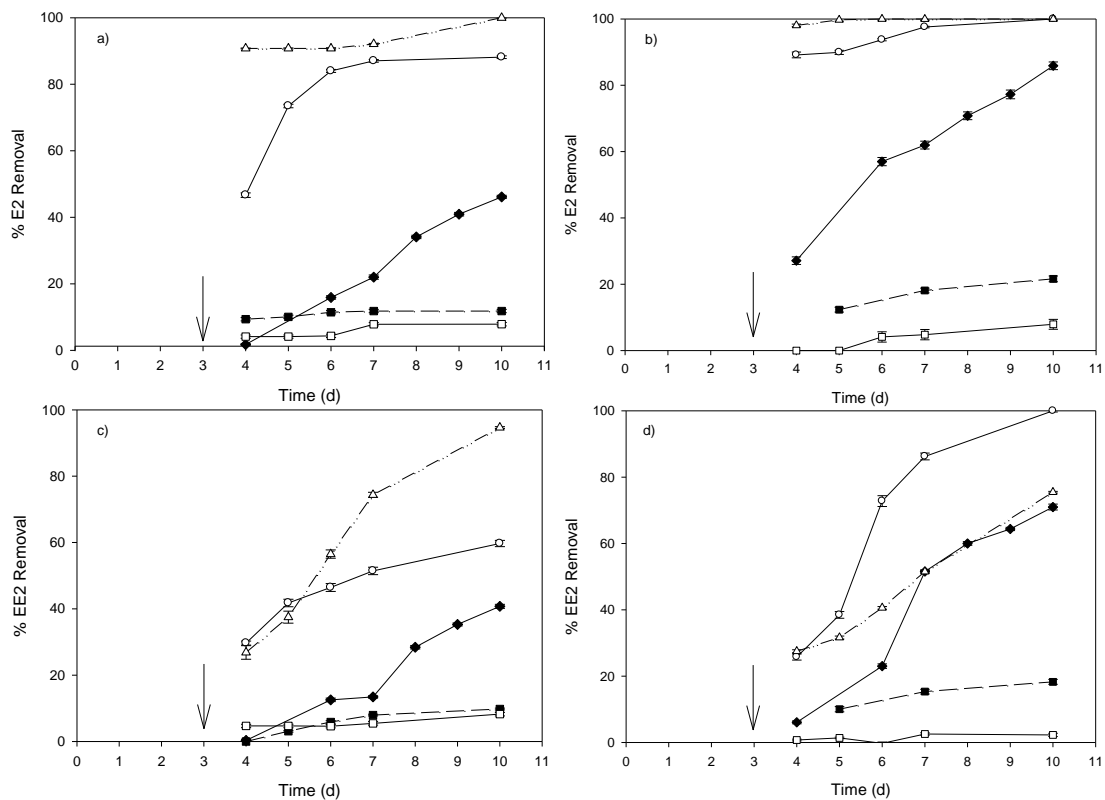


Figure 2 Time-course profile (10 d) of a) %E2 removal by *S.capricornutum* b) %E2 removal by *C.reinhardtii* c) %EE2 removal by *S.capricornutum* d) %EE2 removal by *C.reinhardtii* (B, algae with hormones (\circ); C, killed control (\blacklozenge); D, abiotic culture with hormones (\blacksquare); E, abiotic culture with hormones and light protected (\square); G, ADC culture with hormones (Δ); hormone and/or ADC addition (\downarrow)). Y-error bars represent the standard deviation of three replicates.

The photodegradation (D) of both hormones was low, although it was higher in *C.reinhardtii*. Because the GMs were composed of different compounds, photodegradation could differ and therefore affect hormone removal. Evaporation losses (E) were low for both algae cultures.

In all of the graphs in Fig.2, it can be observed that the removal percentage increased over time. The highest percentages were observed for the E2 removal achieved by both strains, and both were higher than the killed control, which quantified the compound adsorption onto the biomass. Luo et al. (2014) reported a higher adsorption capacity of dead algal cells than live cells due to changes in the cell membrane. At the end of the present study, the adsorption percentages had increased for both strains; as contact time increased, larger quantities were adsorbed. Shi et al. (2010) also reported that a portion of the estrogens was adsorbed on the live algae consortium surface, with great differences between the algal species despite similar initial biomass concentrations. Similar effects were reported for other pollutants such as polycyclic aromatic hydrocarbons (PAHs) tested on *S.capricornutum* and *Chlorella sp.* (Luo et al., 2014). After 24 h, E2 and EE2 adsorption (C) on *S.capricornutum* was negligible (2% for E2 and 0% for EE2), whereas *C.reinhardtii* presented higher values (27% and 6% for E2 and EE2, respectively). The biodegradation percentages for E2 were 62% and 71% for conditions B and G, respectively, and 20% and 22% for EE2. EE2 adsorption onto the algae surface was lower than E2 adsorption; this effect is in accordance with the findings of Shi et al. (2010), who reported that the affinity of EE2 for the algae surface was lower than that of natural estrogens. The results are also in accordance with those obtained when other microorganisms were used, such as fungal biomass (Blázquez and Guieysse, 2008).

Table 2 shows the E2 and EE2 removal percentages at 24 h and 7d after spiking. The hormone removal was related to an increase in biomass, and higher values of removal were achieved when growth increased. This increase occurred when ADC was added (experiment G), which enhanced *S.capricornutum* growth considerably (Fig.1). These percentages (>95%) were higher than the ones previously published by Shi et al. (2010), who reported a maximum percentage of 54% for E2 removal. On the other hand, *C.reinhardtii* E2 removal was similar with and without

ADC (experiment G and B) because the growth was exponential at the beginning due to assimilation of nutrients present in the culture. On the contrary, no increase in biomass was observed when ADC was added to the *S.capricornutum* culture. Twenty-four hours after ADC addition, E2 removal was similar for both strains: 0.012 mg E2_{removed}/mg biomass for *S.capricornutum* and 0.014 mg E2_{removed}/mg biomass for *C.reinhardtii*. Based on these results, it can be confirmed that E2 removal is related to biomass.

Table 2 E2 and EE2 removal percentages for *S.capricornutum* and *C.reinhardtii* 24 h after spiking and at the end of the experiment, 7 days after spiking. (B, algae with hormones; C, killed control; G, ADC culture with hormones).

	<i>Selenastrum capricornutum</i>				<i>Chlamydomonas reinhardtii</i>			
	<u>E2 (%)</u>		<u>EE2 (%)</u>		<u>E2 (%)</u>		<u>EE2 (%)</u>	
	<u>24h</u>	<u>7d</u>	<u>24h</u>	<u>7d</u>	<u>24h</u>	<u>7d</u>	<u>24h</u>	<u>7d</u>
B	47	88	30	60	89	100	26	100
C	2	46	0	41	27	86	6	71
G	91	100	27	95	98	100	28	76

EE2 is more stable than E2 and more persistent on the environment (Rudder et al., 2004), so it is more difficult to remove by both strains. For experiment G, EE2 removal per mg of biomass was lower compared with E2 removal for both strains. This result is in accordance with Nadal et al. (2006), who found that the photodegradation rate was highly dependent on the molecular weight (MW), in the present work EE2 has a higher MW than E2. The EE2 removal values were 0.004 and 0.003 mg EE2_{removed}/mg biomass for the *S.capricornutum* and *C.reinhardtii* cultures, respectively. There were no significant differences in hormone removal between the experiments with and without ADC for both strains.

At the end of the experiment, E2 was completely removed by both strains, except by *S.capricornutum* in condition B (88%), where it seems that the biomass was not enough to remove E2 completely. The final EE2 removal percentages were consistent with the amount of biomass present. *S.capricornutum* removed a higher percentage of EE2 as biomass increased, in both the B and G experiments, as did *C.reinhardtii*, which was capable of removing EE2 completely without an additional nutrient supply.

3.3 Endocrine disruptor degradation

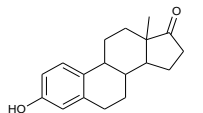
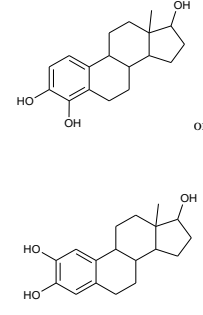
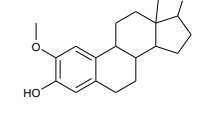
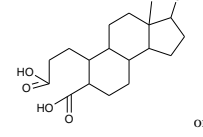
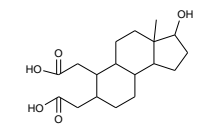
Twenty-six endocrine disruptors and related compounds were analysed in conditions F and G at the time of spiking and at the end of the experiment. The results can be found in Fig.SF1(SM).

Only two of the compounds analysed, tris(2-butoxyethyl)phosphate (TBEP) and bisphenol A (BPA), were detected in the cultures with ADC (F and G) (see Figure SF1 in S2). *C.reinhardtii* achieved TBEP removals of 15% and 22% in the presence and absence of E2 and EE2. On the other hand, TBEP was not significantly removed by *S.capricornutum* when E2 and EE2 were spiked, but it was removed below the method limit of detection in non-spiked samples. This is the first study reporting the removal of TBEP by microalgae. Therefore, further studies should be conducted to further investigate this phenomenon, although possible interactions between TBEP and hormones could have occurred. The final concentrations of BPA were below the method limits detection, so both strains were able to remove it. The results for BPA degradation by algae are in accordance with previous algal studies, such as the one by Gattullo et al. (2012), who reported 48% removal of BPA (4 mg/L initial concentration) with *Monoraphidium braunii* after 4 days of treatment. Li et al. (2009) performed similar experiments and achieved 88% removal with the lowest concentration tested (0.01 mg/L BPA) in a 16-day treatment with *Stephanodiscus hantzschii*.

3.4 Transformation products identified during algae treatments

Information about the degradation by-products of E2 and EE2 that is available in the literature was compiled to create an in-house library (as indicated in materials and methods section). This library (Table ST1, SM) was used to perform a screening analysis of the samples treated with algae using a LC-LTQ-Orbitrap. Those TPs tentatively detected in the samples together with their molecular mass and chemical structure are listed in Tables 3 and ST (SM).

Table 3 Transformation products of E2 and EE2 detected during the 7 batch experiments with the two algae tested (x: Experiments in which the transformation product was detected). More detailed information about the TPs can be found in Table ST1 (SM).

TP	Molecular structure	<i>Selenastrum capricornutum</i>							<i>Chlamydomonas reinhardtii</i>									
		A	B	C	D	E	F	G	A	B	C	D	E	F	G			
1 (E2 TP)			x				x		x					x				x
2 (E2 TP)			x	x	x				x									x
3 (E2 TP)			x						x									
4 (E2 TP)			x	x					x									x
5 (E2 TP)			x	x					x									x

compound was only detected during the experiments carried out with *S.capricornutum* in samples B and G.

Concerning the degradation of EE2, 6 TPs were tentatively proposed (section S3, SM). TP6 was postulated as a transformation product in sample B in the experiments with *C.reinhardtii*. This compound was also detected, among other transformation products, by Della Greca et al. (2008) during degradation studies of EE2 with different microalgae, including *S.capricornutum*.

However, in our study with *S.capricornutum*, it was not possible to detect this compound. The difference in these results may be due to the different concentration of EE2 used during the experiments (5 mg/L in the present work vs. 100 mg/L by Della Greca et al. (2008)). TP7 was also postulated by Della Greca et al.(2008) as an EE2 biodegradation product of algae; in our study, however, it was detected in the killed control (C) with both algae strains, indicating the involvement of processes other than algae degradation. TP8, TP9, TP10 and TP11 were detected under different experimental conditions, with and without algae, with different results for each strain. The GM composition can lead to the formation of different radicals that identify different TPs in each culture. Luo et al. (2014) grew two strains in the same GM and found the same TPs, concluding that the identification was independent of the strain. The presence of some of those TPs in the experiment C (killed control) suggests that the organism responsible for its generation was not only the algae.

4 Conclusions

Microalgae growth was enhanced during the treatment of ADC due to the nutrient supply provided by such effluent. ADC addition also enhanced hormone elimination since removal is directly related to the biomass in the culture; 89% biodegradation of E2 was observed 24 h after ADC was added in the *S. Capricornutum* culture. E2 and EE2 were shown not to be toxic for microalgae growth, at the concentration tested, and both strains were able to remove both

estrogenic compounds within 7 days, being EE2 the most recalcitrant one. The final removal values for both compounds were 88-100% when treated with *S.capricornutum* and between 76-100% when treated with *C.reinhardtii*. Several degradation products of E2 and EE2 were detected in the samples treated with the algae although only one TP directly related to *S.capricornutum* degradation of E2, and another related to EE2 degradation in the *C.reinhardtii* culture. A set of TPs also detected in the treated samples, were attributed to photodegradation processes. Moreover, BPA was completely removed in ADC treated by both strains, both , with and without the addition of hormones in the experiments whereas TBEP was only degraded when no hormones were added.

Acknowledgments

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Appendix A. Supplementary material

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SUPPLEMENTARY MATERIAL

Microalgae cultivation on wastewater digestate: β -Estradiol and 17α -Ethinylestradiol degradation and transformation products identification

Andrea Hom-Díaz^a, Marta Llorca^b, Sara Rodríguez-Mozaz^b, Teresa Vicent^a, Damià Barceló^{b,c}, Paqui Blánquez^{a,*}

^a Chemical Engineering Department, Escola d'Enginyeria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

^b Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, C/Emili Grahit, 101-E17003 Girona, Spain

^c Water and Soil Quality Research Group, Department of Environmental Chemistry (IDAEA-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

*Corresponding author: Paqui Blánquez

Departament d'Enginyeria Química
Escola d'Enginyeria
Universitat Autònoma de Barcelona
08193 Cerdanyola del Vallès. Spain
E-mail address: paqui.blanquez@uab.cat

Tel: +34 93 581 18 79; Fax: +34 93 581 20 13

SUPPLEMENTARY MATERIAL

S1. Experimental set up

All of the experiments were carried out in 100 mL Erlenmeyer flasks containing an initial GM volume of 50 mL. Axenic culture (A) and ADC plus hormones culture (F) were compared with experimental conditions B and G, respectively, for spiked hormone removal evaluation. A killed control (C) was performed to assess the contribution of sorption to the overall removal of hormones, in which algae were thermally inactivated (20 minutes at 121°C). Two abiotic controls were carried out (D and E) containing sterilised medium without algae; they helped to determine whether light, evaporation and other physico-chemical processes had any effect on estrogen removal. E was covered to be light preserved so that the contribution of

photodegradation to the hormones removal could be determined. Unitary samples were taken daily and analysed.

S2. Analytical methods for the identification of transformation products

The samples were analysed using an on-line turbulent flow chromatography system coupled to a hybrid linear ion trap – high resolution mass spectrometer LTQ Orbitrap (TFC-LTQ Orbitrap). An Aria TLX-1 chromatographic system (Thermo Fisher Scientific) was used for purification and separation purposes. This system was composed of a PAL auto sampler and two mixing quaternary pumps (eluting pump and loading pump). The entire system was controlled via Aria software, version 1.6, under Xcalibur 2.2 software. The compounds were extracted using on-line turbulent flow chromatography (TFC) based on an earlier published work by Gorga et al. (2013) for the analysis of endocrine disruptors. The on-line extraction was performed in a Cyclone chromatographic column (50×0.5 mm, 60 μ m particle size, 60 Å pore size; Thermo Fisher Scientific, Franklin, MA), and the compounds were separated using a Hypersil GOLD analytical column (50×2.1 ; 3 μ m; Thermo Fisher Scientific, Franklin, MA). The extraction process consisted of two main steps using the Focus Mode operative mode. First, 10 μ l of sample was introduced into the TFC column at 1.5 ml/min with acidified water with formic acid (0.1%), where the analytes of interest were retained in the active pore sites while the rest of the matrix was discharged to the waste. In the second step, the compounds were desorbed from the TFC column onto the analytical column through a normal LC gradient with water (0.1% formic acid) and acetonitrile. The total run time for each injection was 10 min.

The chromatograph was coupled to a hybrid linear ion trap-Fourier Transform Mass Spectrometry analyser (LTQ-Orbitrap VelosTM, Thermo Fisher Scientific), equipped with a diverter valve (used to divert to waste unwanted portions of chromatographic runs) and an Electrospray Ionisation (ESI) source. The diverter valve was used with three valve positions:

from 0 to 1.5 min, the flow was discharged to the waste; from 1.50 to 7.75 min, the valve was switched to injection mode; and from 7.75 to 10.00 min, the valve was switched again to the waste. The ionisation of the compounds was performed in the negative mode after a pre-screening in the positive mode in which no different chromatographic peaks were detected. Mass calibration and mass accuracy checks were performed prior to every sample run with LTQ ESI Negative Ion Calibration Solution (Thermo Fisher Scientific), and the mass accuracy was always within an error of ± 5 ppm. The ionisation voltage was set at 3 kV with the sheath gas flow at 35, auxiliary gas flow at 10, S-Lens RF level at 60% and the capillary temperature and the source heater temperature at 450 °C. The samples were acquired using two different acquisition methods in parallel: 1) the first method was triggered through full scan within a mass-to-charge (m/z) range of 100 to 700 m/z at a resolving power of 60,000 FWHM. 2) The second experiment was performed using a data-dependent analysis based on the MS fragmentation of the m/z detected in the 1st experiment that coincided with the series of masses listed in the in-house library built by the authors. The ions were isolated in the ion trap with a width of 2.0 Da, and the collision induced a dissociation activation type (Q = 0.250 and an activation time of 30 ms), with normalised collision energy (35) detected at an HRMS of 7,000. Xcalibur 2.2 (Thermo Scientific) software was used for data interpretation.

Data processing was carried out with Exact Finder 2.5 software, which was used to automatically detect the compounds described in the literature and listed by the authors in an in-house library. The identification of compounds was based on double bond equivalents (DBE), with errors within ± 5 ppm; when possible, the MS2 fragmentation was interpreted for each compound.

S3. Estrogenic compounds degradation

The 26 endocrine disruptors and related compounds analysed in conditions F and G are listed: caffeine, 1H-benzotriazole, 5-methyl-1H-benzotriazole, tris(2chloroethyl)phosphate,

tris(chloroisopropyl)phosphate, levonorgestel, progesterone, tris(2-butoxyethyl)phosphate (TBEP), estriol-3-sulfate, estriol-16-glucuronide, estrone-3-sulphate, methylparaben, estriol, estrone-3-glucuronide, estradiol-17-glucuronide, ethylparaben, propylparaben, bisphenol A (BPA), benzylparaben, estradiol, estrone, diethylstilbestrol, trichlorocaraben, triclosan, octylphenol and nonylphenol. Fig. SF1 present the results of the detected compounds, TBEP and BPA.

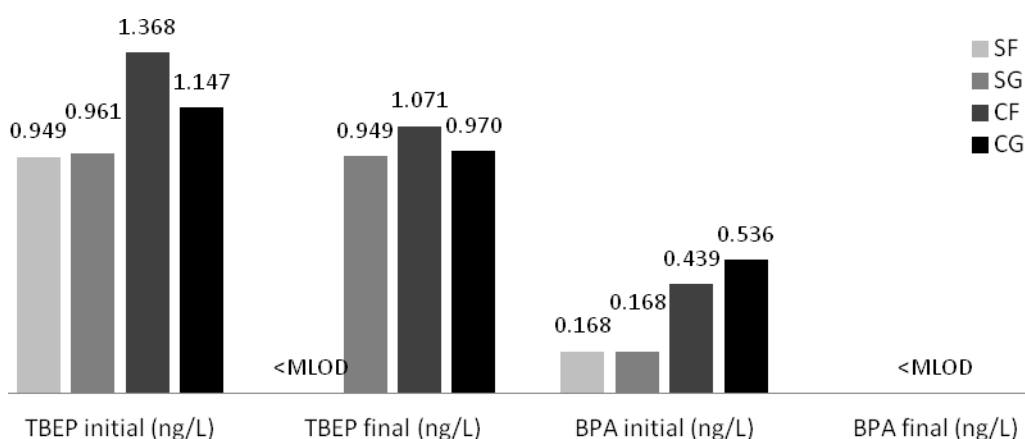


Figure SF1 TBEP and BPA removal by microalgae (SF: *S. capricornutum* not spiked with hormones; SG: *S. capricornutum* spiked with hormones; CF: *C. reinhardtii* not spiked with hormones; CG: *C. reinhardtii* spiked with hormones; MLOD: Method Limit of Detection)

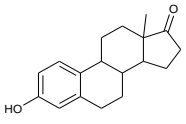
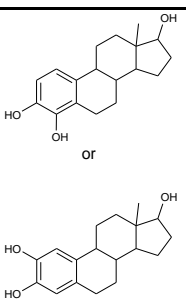
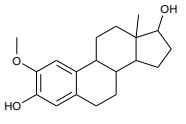
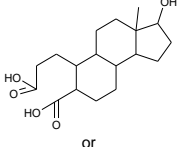
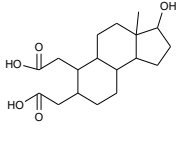
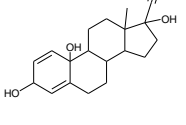
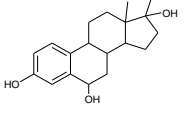
S4. Transformation products identified during algae treatments

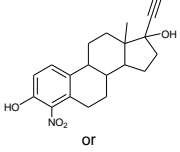
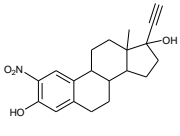
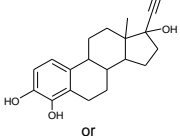
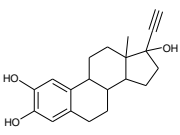
TP1 results from the oxidation of the alcohol at position 17. In this case, the compound was completely identified by comparing it (retention time and spectrum) with the standard compound. Estrone was identified as a degradation product of estradiol in algae cultures (Lai et al., 2002) and in different biological processes such as anaerobic degradation (Czajka and Londry, 2006), degradation by proteobacterias (Pauwels et al., 2008), degradation by organisms present in water and activated sludge (Sarmah and Northcott, 2008; Ternes et al., 1999) and

non-biological processes such as photolysis or oxidation (Mazellier et al., 2008; Zhao et al., 2008). TP2 originated from the hydroxylation of the aromatic ring during the treatments. However, it was not possible to identify the oxidation position, and two configurations were postulated for this compound (Table 3 and ST1). TP2 was detected in previous studies performed with the fungi *Aspargillus alliaceus* (Williamson et al., 1985), as well as in ozonation experiments (Bila et al., 2007; Maniero et al., 2008). TP4 and TP5 were identified as possible transformation products in our study because two different chromatographic peaks (4.50 min and 5.00 min) were detected, in both algae experiments, for samples B and G, as well as in the killed control (C). However, it was not possible to differentiate between TP4 and TP5. The fragmentation patterns (during MS2 experiments) of these two compounds were exactly the same, as was their retention time according to the log P calculated by VCCLAB (Tetko et al., 2005; VCCLAB, 2005) (Table ST1). TP4 and TP5 were identified as ozonation products in a previous work developed by Irmak et al. (2005).

TP8 and TP9 have previously been reported to be transformation products of EE2 obtained via non-biotic nitration by ammonia-oxidising bacteria in studies carried out by Gaulke et al. (2008) and in various biotic and non-biotic degradation studies performed by Skotnicka-Pitak et al. (2008). TP10 and TP11, corresponding to a mass of 311.1690 ([M-H]⁻), were detected, and their structure has been described in previously published works. However, it was not possible to distinguish the compounds by MS2 pattern or by their chromatographic retention time due to the similarity between the log P calculated by VCCLAB (Tetko et al., 2005; VCCLAB, 2005). Nonetheless, both compounds were detected in the experiments performed with *C. reinhardtii* (experiments B and G) as well as in experiment C with both algae. This last experiment could indicate that the generation of these compounds is not only due to the algae as Yi and Harper Jr. (2007) postulated in their nitrification studies.

Table ST1 Transformation products of E2 and EE2 detected during algae experiments

TP	Molecular structure	log P (VCCLA B, 2005; Wiley et al., 2000)	± SD	tr (min)	Molecular formulae	Exact mass	Error (ppm)	Organism or process
1 (E2 TP)	Estrone 	3.65	0.32	7.06	C ₁₈ H ₂₂ O ₂	270.1660	1.924 - 4.045	i) <i>Chlorella vulgaris</i> (Lai et al., 2002) ii) Anaerobic degradation in lake sediment or sludge (Czajka and Londry, 2006) iii) The strains belong to the α, β and γ-Proteobacteria (Pauwels et al., 2008) iv) Groundwater and river water biodegradation (Sarmah and Northcott, 2008) v) Activated sludge (Ternes et al., 1999) vi) Photolysis (Mazellier et al., 2008) vii) Oxidation (Zhao et al., 2008)
2 (E2 TP)	 or	3.52	0.30	6.00	C ₁₈ H ₂₄ O ₃	288.1770	1.899 - 3.772	i) Ozonation (Bila et al., 2007; Maniero et al., 2008) ii) <i>Aspergillus alliaceus</i> (Williamson et al., 1985)
3 (E2 TP)		3.77	0.26	6.30	C ₁₉ H ₂₆ O ₃	302.1930	2.906 - 3.615	Metabolites of estradiol (Dubey et al., 2001; Xiao et al., 2001)
4 (E2 TP)	 or	2.27	0.51	4.50	C ₁₈ H ₂₄ O ₅	320.1670	2.140 - 3.038	Ozonation and O ₃ /UV (Irmak et al., 2005)
5 (E2 TP)		2.29	0.55	5.00	C ₁₈ H ₂₄ O ₅	320.1670	2.072 - 3.038	
6 (EE2 TP)		2.14	0.71	5.22	C ₂₀ H ₂₄ O ₃	311.16526	1.030	<i>Selenastrum quadricauda</i> (Della Greca et al., 2008)
7 (EE2 TP)		2.89	0.51	5.80	C ₂₀ H ₂₄ O ₃	311.16526	2.359 - 3.874	<i>Ankistrodesmus braunii</i> (Della Greca et al., 2008)

8 (EE2 TP)	 OR	4.16	0.46	7.20	C ₂₀ H ₂₃ O ₄ N	340.15543	1.957 - 3.034	i) <i>N. europaea</i> and <i>Nitrosospira multiformis</i> bacteria (Gaulke et al., 2008)
9 (EE2 TP)		4.16	0.53	7.70	C ₂₀ H ₂₃ O ₄ N	340.15543	2.316 - 3.213	ii) Biotic and abiotic degradation studies (Skotnicka-Pitak et al., 2008)
10 (EE2 TP)	 OR	3.67	0.56	6.23	C ₂₀ H ₂₄ O ₃	311.16526	2.209 - 3.677	Nitrification processes (Yi and Harper Jr., 2007)
11 (EE2 TP)		3.63	0.53	6.61	C ₂₀ H ₂₄ O ₃	311.16526	2.239 - 3.972	

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