Effect of cultivation conditions on β-estradiol removal in laboratory and pilot-plant photobioreactors by an algal-bacterial consortium treating urban wastewater

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Abstract

The use of microalgal consortia for urban wastewater treatment is an increasing trend, as it allows simultaneous nutrient removal and biomass production. Emerging contaminants proposed for the list of priority substances such as the hormone 17β-estradiol are commonly found in urban wastewater, and their removal using algal monocultures has been accomplished. Due to the inherent potential of algae-based systems, this study aimed to assess the capability of native photobioreactor biomass to remove 17β-estradiol under indoor and outdoor conditions. At the same time, the microbial community changes in regular and bioaugmented operations with *Scenedesmus* were assessed. The results show that almost complete removal (>93.75%) of the hormone 17β-estradiol can be attained in the system under favourable seasonal conditions, although these conditions greatly influence biomass concentrations and microbial diversity. Even under the harsh conditions of low temperatures and solar irradiation, the established consortium removed more than 50% of the pollutant in 24 h. While species from genus *Chlorella* were stable during the entire operation, the microbial diversity analysis revealed that assorted and evenly distributed populations stimulate the removal rates. Bioaugmentation assays proved that the input of additional biomass results in higher overall removal and decreases the yield per mg of biomass.

Keywords:

Hormones; microalgae; temperature; photobioreactor; bioaugmentation; microbial diversity.

1 Introduction

Microalgal culturing is gradually increasing worldwide due to the possibility of using algal biomass as a biofuel feedstock because of its high content of lipids, proteins and carbohydrates (Salama et al., 2017). Microalgae are photosynthetic microorganisms that can be used in wastewater (WW) treatment since they are able to utilize CO_2, organic carbon (mixotrophic growth) and inorganic nutrients (Gonçalves et al., 2016; Judd et al., 2015). Moreover, consortia present in open microalgal systems have great biotechnological potential since nutrient removal and biomass production are achieved at the same time (Ramanan et al., 2016).

The idea of using microalgae in WW treatment has been considered for decades. Currently, several studies have demonstrated the capacity of microalgal-based processes to treat effluents from different origins, including anaerobic digesters, and to produce biomass for a variety of uses (Gonçalves et al., 2016; Uggetti et al., 2014). The reported data demonstrate that high nutrient removal rates and biomass production can be achieved in systems harbouring microalgal-bacterial consortia. Seasonality is relevant to achieving adequate nutrient removal efficiencies in microalgal-based processes. These processes are affected by temperature, daylight duration and intensity, and biomass growth, which are four important factors that directly influence biodegradation, photodegradation, volatilization and the sorption of nutrients and pollutants (Garcia-Rodríguez et al., 2014; Lee et al., 2015).

Effluents are typically rich in nutrients and contain micropollutants, which have not received much attention from researchers in the microalgal-based systems field. The hormone 17β-estradiol (E2) is a widespread emerging contaminant (EC) detected in urban and industrial WW (Santos et al., 2010). Due to its environmental fate and toxicological effects, E2 has been proposed by the EU commission, together with

diclofenac and 17-α-ethinylestradiol (EE2), to be included in the list of priority substances. Furthermore, these substances have been included in the watch list of substances for Union-wide monitoring in the field of water policy (Carvalho et al., 2015). Although the concentrations currently detected in wastewater are in the range between ng and µg, they can induce adverse ecological effects on organisms, and can act as endocrine disruptors (Jobling et al., 1998).

Although microalgal pure cultures have been applied for nutrient and EC removal in different WW (Hom-Diaz et al., 2015; Peng et al., 2014), it is difficult to maintain a microalgal monoculture in open processes. Accordingly, several studies have reported the advantages of co-cultivated photosynthetic microorganisms in WW treatment. On the one hand, Koreivienė *et al.* (Koreivienė et al., 2014) reported high nutrient removal efficiencies in municipal WW treatment using a non-native consortium composed of *Chlorella* sp. and *Scenedesmus* sp. On the other hand, Chinnasamy *et al*. (Chinnasamy et al., 2010) obtained a high biomass and lipid production potential using a native microalgal consortium. Finally, Renuka *et al. (Renuka et al., 2013)* described the self-flocculating potential of native and non-native consortia in the treatment of a primary-treated sewage effluent. Among the possible combinations of microalgae, many consortia involve *Chlorella* and *Scenedesmus* species (Koreivienė et al., 2014; Renuka et al., 2013; Whitton et al., 2016). Moreover, few woks have been carried out in order to study the biodegradadion of 17β-estradiol at pilot-plant photobioreactor, and in this study the capability of native photobioreactor biomass treating urban wastewater to remove at indoor and outdoor conditions has been assessed. Outdoor conditions studies are important in order to evaluate how environmental conditions can affect the system.

Specifically, this work focuses on (i) the comparison of E2 removal by a pilot PBR biomass under indoor and outdoor conditions, (ii) the evaluation of the community changes in the pilot PBR and (iii) the evaluation of E2 removal after bioaugmentation with a *Scenedesmus* sp. culture.

2. Materials and methods

2.1. Chemicals and Inoculum

The 17β-estradiol (E2) reagent (>98% purity) was purchased from Sigma-Aldrich (Barcelona, Spain). The 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 Invitrogen (Barcelona, Spain), Sigma-Aldrich (Barcelona, Spain) and Panreac (Barcelona, Spain).

The inoculum of the microalgal photobioreactor was collected from a lake described elsewhere (Hom-Diaz et al., 2017).

2.2. Microalgal pilot photobioreactor

The microalgal photobioreactor (PBR) was located on the roof of the Chemical, Biological and Environmental Engineering Department at the Universitat Autònoma de Barcelona (Barcelona, Spain). The experimental design and setup and information on the PBR that was operated for a year treating toilet wastewater (WW) were previously reported by Hom Diaz *et al.* (Hom-Diaz et al., 2017). During the first period (A), the PBR was working at a hydraulic retention time (HRT) of 8 days; during the second and third periods (B and C, respectively), the HRT was increased to 12 days. The E2 spiking experiments were conducted during the steady state of each operation. These periods of time correspond to different seasons of the year, affecting to the biomass concentration in the photobioreactor due to differences in the temperature and solar irradiance. Additional information is available in Hom-Díaz et al., 2017. The PBR was spiked at a

concentration of 2 mg E2/L. The PBR characteristics prior to the assays are presented in Table 1.

2.3. Field experiments

Batch experiments were conducted in the pilot PBR by stopping the wastewater influent supply and adding 2 mg/L of β-estradiol (E2) (no effluent withdrawal) into the pilot PBR. The concentration was monitored during 24 h, and 500 mL samples were regularly withdrawn for subsequent analysis before normal continuous operation was resumed.

Spike experiments were conducted at the end of each operational HRT. The PBR-A was carried out after sunset, whereas the PBR-B and C experiments were started after the sunrise. Detailed information on the samples taken is shown in Table S1 (Supplementary Material (SM)). The environmental parameters of each assay are presented in Table 2.

Samples for the microbial diversity analysis were taken before the spiking and 24 h after the spiking was conducted. The processing and analysis methodologies are described in section 2.6.

2.4. Batch experiments at laboratory scale

The batch assays (E2-A, E2-B and E2-C) (Tables 1, 3 and S1) were conducted in parallel with the respective field experiments (PBR-A, PBR-B and PBR-C) and using the PBR biomass that was incubated indoors; again, 2 mg/L of the E2 were spiked. These batch experiments were carried out in 250 mL Erlenmeyer flasks incubated in agitation at 130 rpm (orbital shaker Kuhner, LS-X, Switzerland) at 25±2°C and under continuous fluorescent lamp irradiation (172±18 mmol/m^2 s) or complete darkness (flasks covered with foil). The irradiance level was measured with a light metre (LI.189,

LI-COR Quantum/Radiometer/Photometer, USA). Killed controls (autoclaved 121°C, 30 min) were also included, and all tests were conducted in triplicate.

Additionally, a bioaugmentation assay (BA) was conducted (E2-D) at the same time as the E2-C. The PBR biomass was mixed with the microalgal biomass coming from an industrial photobioreactor (IPBR) where *Scenedesmus* sp. was dominant. Two bioaugmentation ratios (v/v) were evaluated: 3:1 and 1:1 (PBR biomass:IPBR biomass). All the batch experiments (Table 3) were conducted individually under light and dark conditions.

2.5. Analysis

The E2 and estrone (E1) concentrations were quantified following the methodology proposed by Blánquez and Guieysse (Blánquez and Guieysse, 2008) using a Dionex 3000 Ultimate HPLC system (Barcelona, Spain) equipped with an UV detector at 220 nm. The column temperature was 30°C, and a sample volume of 20 µL was injected from a Dionex autosampler. Chromatographic separation was achieved using a Phenomenex Kinetex C-18 column. The elution was carried out at 1 mL/min using a 60:40 ultrapure water (pH 7)/acetonitrile (vol.) mobile phase. The E2 retention time was 15 min and 14 min for E1, and the limit of quantification was 0.125 mg/L for both hormones.

The pH and temperature were recorded in situ with a PCE-PHD 1 multimeter (PCE Instruments, Albacete, Spain). The total suspended solids (TSS) and chemical oxygen demand (COD) were analysed following the protocol of standard methods (APHA et al., 1999). The total and soluble ammonia (NH_4^+) and phosphorus (P) were measured using the Y15 Analyzer (Biosystems, Barcelona, Spain).

2.6. Microbial diversity

2.6.1 Total DNA extraction

Fifty millilitres of each sample were filtered in 0.22 µm GV Durapore® membrane filters (Millipore) right after collection and stored at -80°C until further processed altogether. The total DNA extraction was conducted using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc.) following the manufacturer instructions.

2.6.2 PCR amplification

For the eukaryotic community analysis, a 560-bp fragment from 18S rRNA gene was amplified using the primer set Euk1A and Euk516r (Díez et al., 2001) with a GC-clamp attached to the 5' end of the reverse primer. The PCR reactions (50 µL) contained (final concentrations) 1x PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 0.5 µM of each primer and 2.5 U of *Taq* DNA polymerase and 10 ng of total DNA. The amplification programme consisted of initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 60 s; and a single final extension step at 72°C for 5 min. To assess the cyanobacterial diversity, a fragment (450 bp) of its 16S rRNA gene was amplified with the primer set CYA359f and CYA781r (Nübel et al., 1997). In this case, the GC-clamp was attached to the 5' end of the forward primer. The PCR reactions were carried out as described above with the following amplification programme: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and a single final extension step at 72°C for 5 min. All PCR amplicons were checked for the correct size in 1.5% agarose gel, stained with ethidium bromide, and quantified with Quantity One 1-D Analysis Software (Bio-Rad) using a low DNA mass ladder (Invitrogen). Detailed information regarding the oligonucleotide sequences used is presented in Table 4.

2.6.3 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with the Dcode Universal Mutation Detection System (Bio-Rad). The PCR products were loaded directly onto 6% (wt/vol) polyacrylamide gels containing a 20–45% (Eukarya) or 30–70% (Cyanobacteria) chemical denaturing gradient and run in 1x TAE (40 mM Tris acetate [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gradients were formed with 6% (wt/vol) acrylamide stock solutions (acrylamide-N:N'-methylenebisacrylamide, 37:1) where the 100% denaturing solution contained 7 M urea (Bio-Rad) and 40% [vol/vol] formamide (Merck) deionized with AG501-X8 mixed-bed resin (Bio-Rad). The electrophoresis was performed at a constant voltage of 75 V and a temperature of 60°C for 16 h. After electrophoresis, the gels were stained during 30 min in Milli-Q water containing ethidium bromide (0.5 mg/L), rinsed for 30 min with Milli-Q water, and photographed with UV transillumination (302 nm) with the Universal Hood II (Bio-Rad). The fingerprinting profiles were processed using InfoQuest™ FP version 4.5, and subsequent analysis and statistics were performed using R version 3.3.2.

2.6.4 Sequencing

Prominent bands were excised using micropipette tips, placed inside Eppendorf tubes containing 15 µL of MilliQ water and stored at 4°C for 24 h to allow DNA diffusion before re-amplification. The amplified DNA fragments from the gel bands were placed in 96-well plates and sequenced at external facilities by Macrogen, Inc. (South Korea). The sequences were manually trimmed and quality checked using FinchTV 1.4.0 (Geospizza, Inc.) and further checked for chimaeras using the UCHIME algorithm (Edgar et al., 2011). The curated sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers

KY076627—KY076664 and KY073310—KY073315 for Eukarya and Cyanobacteria, respectively.

2.7. Experimental replication and statistical treatment of data

The batch assays (E2-A, E2-B and E3-C) were conducted under the experimental conditions described in section 2.4, killed controls were also included and all tests were conducted in triplicate. Standard deviations are presented in Figures 2 and 5. The fingerprinting profiles of the DGGE described in section 2.6.2 include two biological replicates for each assay and were processed using InfoQuest™ FP version 4.5. Subsequent analysis and statistics were performed using R version 3.3.2.

3. Results and discussion

3.1. E2 removal batch experiments in the pilot PBR

Removal of β-estradiol (E2) and appearance of estrone (E1) were evaluated during the assay. It has been reported that under aerobic or anoxic conditions, E2 could be first oxidized to E1, further oxidized to unknown metabolites, and finally oxidized to CO_2 and water (Shi et al., 2010). The removal of E2 and formation of E1 over time and during the three assays (PBR-A, PBR-B and PBR-C) are shown in Figure 1. The three assays followed similar trends in terms of E2 removal and E1 formation, despite the differences in the final concentration. E2 was not detected at the end of the PBR-A, and most of the E2 removal occurred during the night (81%). While the other two assays, PBR-B and C, had lower final removal percentages (77 and 55%, respectively), similar behaviours were still observed during the three assays. Rapid removal rates were observed in the first 10-13 h, followed by slower removal rates until the end of each experiment.

Information regarding E2 removal using other biological techniques is scarce, although there are some works where bacteria and/or algae have been used and obtain similar E2 and E1 removal percentages in continuous systems (Servos et al., 2005; Shi et al., 2010).

E1 has been reported as a by-product of biodegradation of E2 by the microalgae *Chlorella vulgaris* (Lai et al., 2002), although it can also be formed due to oxidation processes (Servos et al., 2005; Zhao et al., 2008). In these E2 oxidation processes, the concentration of E1 increases, and it is further removed from the treatment system. The mineralization of E1 by bacteria naturally found in rivers was proved by Jürgens et al. (Jürgens et al., 2002) without the appearance of other major degradation products. In this study, the PBR-A showed a rapid E1 formation followed by a constant concentration (Figure. 1A). Inversely, the E2 concentration decreased until it was undetectable at approximately 12 h, when the E1 reached its maximum value.

In the PBR-B and C assays (Fig. 1B and C), E1 was formed at the same rate as E2 disappeared, and a rapid conversion occurred at the beginning. Interestingly, after 5 h, the transformation rate decreased in both assays, and E1 formation was still taking place at the end of the experiment since E2 was not completely removed.

Figure 1-D presents E2 removal for all 3 PBR experiments (A, B and C). Considering TSS concentration and removal results from each assay, more removal occurred as more biomass was present (see Table 1). The determination of the E2 concentration took into account the adsorbed hormone onto the biomass, since an extraction was conducted.

The E2 concentration data were well described by a first order kinetic model (Table 5). Higher reaction rate (k) values indicate faster E2 removal (PBR-A).

The PBR-B and PBR-C had similar TSS concentrations (198 and 162 mg/L, respectively), and the removal percentage was lower on the PBR-C, which indicates that

environmental parameters influenced the system. Some seasonal differences (maximum and minimum temperatures and global solar irradiance (GSI)) among the assays are shown in Table 2. Vader *et al.* (Vader et al., 2000) suggested that season and temperature have an effect on nitrification and may therefore result in changes in the ability of treatment systems to remove oestrogenic compounds, so this effect can also have an impact on microalgae species as well as the other microorganisms present in the PBR. Both air temperature and sunlight irradiation decreased between assays (Table 1), which may have reduced the volatilization rate and caused biomass composition changes (Table S3), therefore decreasing E2 removal. Temperature and solar radiation are known to drive the growth of microalgae, so it is likely they are responsible for the composition changes within the photosynthetic species (Lee et al., 2015). However, heterotrophic bacteria can be especially sensitive to irradiation changes as well (Deller et al., 2006), and slight variations in their concentrations might have disrupted some of the microalgae-bacteria interactions already established in the PBR.

3.2. *Laboratory scale E2 removal batch experiments*

Figure 2 shows E2 removal and E1 behaviour at a laboratory scale under light and dark conditions using PBR biomass.

The microalgal biomass experimental conditions (named algae in Figures 2 and S1) followed similar trends in all three experiments during E2 removal. There were no significant differences between light and dark conditions, leading to the conclusion that E2 photodegradation removal did not occur in 24 h.

However, three different behaviours on E1 formation could be seen, depending on the assay. In the first assay (Fig. 2A), E1 was formed 10 h after the spiking, and the concentration rapidly increased in 3 h. Then, E1 remained constant until the end, and no

removal was detected. On the other hand, in the E2-B assay (Fig. 2B), E1 was formed from the beginning of the experiment and then followed the same trend described in the first assay. Interestingly, E1 decreased in samples under light conditions, meaning that the system was also able to remove the compound. Finally, in the E2-C assay (Fig. 2C), E1 was formed 2 h after the spiking and linearly increased for 10 h. In samples under light conditions, E1 was detected at a lower concentration. Overall, E1 was formed in the presence of active algal biomass (not detected in the killed controls) either under light or dark conditions. In view of the previous results and the results obtained in the PBR it is concluded that the adsorption is very fast (Figure 1) but biodegradation is also very fast, because in 10 hours Estrone (E1) is already detected, both in light as in dark live algae experiments, demonstrating that E1 is a transformation product of biodegradation resulting from PBR biomass activity and not a photodegradation transformation product.

Regarding the removal percentages for each assay (Table 5), in the E2-A, 88% E2 removal was achieved in 13 h, then the removal velocity decreased achieving the complete removal in less than 24 h. This profile described a similar trend as the behaviour occurring in the PBR (Fig. 1A). Although the outdoor system had a better performance than the controlled conditions experiment, the E2-A had a constant rate of 0.159 h^{-1} whereas the PBR-A had a higher value of 0.338 h^{-1} (Table 5) (96% E2 was removed in 12 h). The E2 removal percentages were lower during the E2-B assay; 43% removal was achieved in 10 h, and the final value (24 h) remained at 71%. If constant rate values are compared between batch experiments, $k_{E2\text{-}B}$ is lower than $k_{E2\text{-}A}$ (Table 5). Finally, an 88% E2 removal was achieved upon the end of the E2-C experiment (24 h) for the algae condition. There was an increase of E2 removal in comparison with the E2-B and that has been reflected in the constant value rate, $k_{E2\text{-}C}$=0.084 h^{-1}.

To compare the three assays, the E2 removal yield was calculated (Table 5) considering the initial microalgal biomass. The removal yields of the E2-A, B and C were $3.24 \cdot 10^{-4}$, $2.98 \cdot 10^{-4}$ and $4.52 \cdot 10^{-4}$ mg E2/(mg TSS·h), respectively. Sun irradiation may be the major difference among the systems, as well as temperature changes within the 24 h; this effect is clearly seen during the E2-C assay. During the PBR-C assay, temperatures ranged between 2 and 12°C, whereas under controlled conditions, the E2-C temperature was maintained at 25°C and E2 removal percentages were higher (88%) than outdoors (55%) (Table 5). The differences among removal percentages under controlled conditions may be attributed to the microalgae community composition, so they are further discussed in section 3.3.

Few works have previously studied E2 removal using microalgae in batch assays. Shi *et al.* (Shi et al., 2010) conducted a 6-day batch experiment with 6 pure microalgae strains cultured in synthetic WW containing oestrogenic compounds. Approximately 95% of the E2 was removed (100 mg TSS/L), and 60% removal was achieved in 24 h, corresponding to an E2 removal yield of $2.50 \cdot 10^{-10}$ mg E2/ (mg TSS·h). This low removal yield was due to the low initial E2 spiked concentration (1 ng/L), whereas in the present work, the initial concentration was in the order of mg/L. While in Shi *et al.* (Shi et al., 2010) study, the initial biomass consisted of pure microalgae cultures, the present PBR seems to perform better, in terms of E2 removal, due to the combination of microalgae and WW-native communities.

The abiotic and killed controls were performed together with the PBR active biomass, and detailed information can be found in the SM section 2. Twenty-three percent E2 removal was achieved in the abiotic controls. Sorption percentages were different among the assays, ranging from 8 to 50%, suggesting that E2 is more or less likely to be

adsorbed depending on the biomass composition. As Hom-Diaz *et al.* (Hom-Diaz et al., 2015) reported, the difference in algal biomass composition has a direct effect on the adsorption of pollutants.

3.3. Microbial diversity assessment

The eukaryotic diversity throughout the operation of the PBR was assessed by means of DGGE, and samples were taken right before and after 24 h of each spike. A total of 50 prominent bands were recovered, re-amplified, sequenced and quality filtered, ultimately resulting in 26 high quality sequences. From what is observed in the DGGE profiles (Figure 3), no changes occurred after the E2 spiking, meaning that the oestrogenic compound does not have an immediate effect on populations. The analysis of the obtained sequences revealed the presence of 5 different phyla (Chlorophyta, Rotifera, Blastocladiomycota, Ciliophora and Cercozoa) within the Eukarya domain and two unclassified sequences. Briefly, the PBR was dominated by algae from the genus *Chlorella*, which remained present in all the PBR stages, accompanied by *Pseudospongiococcum*, in less abundance. Other abundant phylotypes, only present in some periods, represented protozoa, rotifer or parasitic chytrid species that fed on the algae. It is likely that changes in the microbial community are partially responsible for the differences in E2 removal. Detailed information regarding the phylogenetic affiliation, closest relatives, and sequence similarity of each band is presented in Table 6.

Visual differences were observed in the algal biomass during the operation periods of the PBR. The microalgae biomass for the PBR-A and B was mainly composed of settling flocs, while in the last assay, the PBR-C, the PBR culture contained free microalgae cells, and less settling flocs were present. No filamentous microorganisms were detected in the Eukarya DGGE that could justify the presence of the settling flocs

in the first stages of the PBR. To explain the phenomenon, another DGGE approach was performed with fewer samples from different time points to reveal the cyanobacterial diversity. The DGGE profiles are presented in Supplementary Figure S2, and the sequences obtained from 6 representative bands revealed that the Cyanobacteria found in the PBR belonged to genera *Phormidium* and *Leptolyngbya* (Supplementary Table S2). Hence, the authors note that the filamentous cyanobacteria from the genus *Phormidium* were responsible for the formation of the settling flocs.

As E2 removal yield has been defined as the amount of E2 removed per mg of TSS and per hour, the resulting values give information about biomass compositional changes that may be occurring in the system. To evaluate these differences, species richness, Shannon diversity and Pielou evenness were calculated using the DGGE band profiles and are presented in Supplementary Table S3. The highest richness within the analysed samples (n 9) was observed in both the PBR-A and PBR-C, which also exhibited high diversity. As was expected, evenness followed the same dynamics. The statistical significance was inferred at $p < 0.05$ using the Wilcoxon rank-sum test due to the limited number of replicates. No significant differences were observed between samples A-C, but both were different from B, respectively. Specifically, this pattern was mirrored in the removal yield under laboratory conditions, where B exhibited lower (1.5–1.8 times) values compared to C and A, respectively. These results are in accordance with other studies (Falås et al., 2016; Servos et al., 2005) where a high diversity of microalgae and bacteria was associated with an increased removal of EC and micropollutants, respectively. Although the removal in B was again low at the pilot scale PBR, the results are hard to compare due to seasonal differences. This is evident in

the case of the PBR-C removal yield, which experienced a sharp decrease likely due to a marked fall in the temperature that affected the microalgal biomass.

Taking into account the configuration of the PBR, the most relevant removal processes that may occur in this system are likely sorption, biodegradation, photodegradation and volatilization. Moreover, it has been proven that a consortia of cyanobacteria/microalgae and bacteria can be efficient in the removal of organic and inorganic pollutants and nutrients from wastewater, compared to axenic cultures (Gonçalves et al., 2016). It has been previously described that hydrolytic enzymatic activities of bacteria are accelerated in the presence of algal-associated polymeric compounds (Martinez et al., 1996). This will therefore speed up the recycling of organic nutrients, which, in turn, can either favour algal growth or increase algae lysis and death (Daufresne et al., 2008).

3.4. E2 removal and diversity assessment in laboratory-scale bioaugmentation experiments

A bioaugmentation assay was carried out to evaluate the impact of having a higher biomass density and a more specialized microalgal biomass for E2 removal. In fact, previous authors reported better results on oestrogenic compound removal depending on the microalgae strain used. Peng *et al.* (Peng et al., 2014) noted better results using *Scenedesmus obliquus* on the steroidal hormone progesterone, rather than *Chlorella pyrenoidosa*.

The biomass used for the bioaugmentation assay (E2-D) came from an industrial PBR (IPBR), which was mainly composed of *Scenedesmus* sp. A total of 6 phylotypes were recovered from the DGGE profiles (Table 6 and Figure 4). Five of the phylotypes belonged to the phylum Chlorophyta and the other to Chytridiomycota. The most

abundant phylotype (band M) exhibited a 100% identity with *Desmodesmus armatus*, a ubiquitous alga inhabiting freshwater and soil under a wide range of environmental conditions. *Desmodesmus armatus* is a homotypic synonym of *Scenedesmus hystrix* var. *armatus*. Accordingly, the IPBR biomass was mainly composed (3 out of 6 phylotypes) of *Scenedesmus* (bands J, M, N) and the fungi *Gaertneriomyces* (band O). The latter is a fungal member of the Phylum Chytridiomycota, commonly found in freshwater and in soils alike (Sime-Ngando, 2012). Some of its species are parasites of small marine green algae and diatoms, while in freshwater, they can infect a wide diversity of hosts, including both large- and small-sized algae (Rasconi et al., 2012; Sime-Ngando, 2012)

During the E2-D assay, the biomass from the PBR (without bioaugmentation) was used as a control, which was the same used in the PBR-C and E2-C assays. The initial characteristics from the IPBR and both ratios tested are shown in Table 1. Figure 5 shows the E2 and E1 concentrations for the control and consortia configurations tested (3:1 and 1:1 (v/v), PBR:IPBR) for the bioaugmentation experiments. As the TSS increased, the E2 concentration decreased, which is in accordance with the results from the PBR and laboratory assays. Complete removal was achieved in the presence of the IPBR biomass, whereas the control (without bioaugmentation) only attained 88% removal at the end of the assay. The two phases can be distinguished on the E2 removal, i.e., a rapid decrease between 1 and 2 h and a period of slower decrease up to 24 h. The samples containing the IPBR biomass seemed to decrease in E2 concentration until the end of the experiment, whereas no complete removal was achieved in the control at the end of the experiment. The removal rate for each condition was determined by the k value (Table 5), showing that as the IPBR biomass ratio grew higher, a higher E2 removal rate was attained. The E2 removal yield values were calculated (Table 5) to consider the initial biomass concentration, since it was different for all three conditions.

The highest E2 removal yield ($4.52 \cdot 10^{-4}$ mg E2/mg TSS·h) was obtained for the control biomass (E2-C), although there was not complete removal, followed by a 3:1 ratio and finally a 1:1 ratio. These results show that the PBR biomass was more efficient at removing E2 compared to the IPBR biomass. The E1 was detected in the consortium control (no bioaugmentation) and both bioaugmentation experiments, following a similar trend. However, the maximum E1 concentration in each case was attained at different times and at different concentrations, in accordance to the E2 removal. In the control consortium, the E1 was formed between 0 and 13 h, and the maximum concentration detected was 1 mg/L, then decreasing to 0.6 mg/L. In the 3:1 ratio experiment, the E1 concentration attained the maximum concentration (1.3 mg/L) 13 h after the spiking and then decreased to 0.5 mg/L. The1:1 ratio experiment had the fastest and highest E1 production of the assay. The concentration peak (1.6 mg/L) was reached in only 10 h and was followed by a rapid decrease to 0.5 mg/L at the end of the assay. While bioaugmentation has been used to enhance microalgae with high lipid content in biodiesel production (Kumar et al., 2016), this is the first time microalgae bioaugmentation has been conducted for emerging pollutant removal or WW treatment. Although the use of *Scenedesmus* sp. did not increase the E2 removal yield in this study, previous authors reported the capacity of this genus for oestrogenic compound removal, achieving high removal percentages (88-100%) (Hom-Diaz et al., 2015). The results from this study confirm that the higher the biomass, the faster the E2 removal. Therefore, increasing the biomass through bioaugmentation improves removal via biodegradation and biomass sorption mechanisms, which occur at the same time and cannot be quantified separately.

4. Conclusions

This work has demonstrated that an algal-based system can completely remove E2 (initial concentration 2 mg/L) during indoor and outdoor batch operations. The rapid removal (12-13 h) occurring in both settings, regardless of light or dark conditions, confirms that E2 removal is not achieved only by photodegradation. Furthermore, estrone is always detected as a result of the process. The results have also shown that more E2 degradation is obtained as microalgal biomass increases, although microbial diversity and especially seasonal variations (such as temperature and sun irradiation) influence the system. Specifically, no remarkable differences have been observed considering indoor and outdoor results, except during the PBR-C assay, where low biomass concentrations under unfavourable environmental conditions demonstrated a poor performance. As other authors previously reported, we confirm that high community diversity and evenness have beneficial implications in micropollutant removal. Neither 3:1 nor 1:1 bioaugmentation strategies with *Scenedesmus* could outmatch the native PBR community in terms of yield. The PBR is composed of transiting species that change with time and stable microalgae, such as *Chlorella*, that remain unaffected by seasonal variations. Taken together, these results suggest that high biodiversity with some well-established species is desired before high biomass. Despite the tough environmental conditions (T=2°C), the removal efficiency of the system was always over 50%. Finally, while the precise mechanism of E2 removal in the batch assays remains to be determined, we suggest that further studies should assess the role of bacteria and its diversity in the system.

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References

APHA, AWWA, WPCF, 1999. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC.

Blánquez, P., Guieysse, B., 2008. Continuous biodegradation of 17β-estradiol and 17α-ethynylestradiol by *Trametes versicolor*. J. Hazard. Mater. 150, 459–462. doi:10.1016/j.jhazmat.2007.09.085

Carvalho, R.N., Ceriani, L., Ippolito, A., Lettieri, T., European Commission. Joint Research Centre. Institute for the Protection and the Security of the Citizen., 2015. Development of the first watch list under the environmental quality standards directive. Publications Office.

Chinnasamy, S., Bhatnagar, A., Hunt, R.W., Das, K.C., 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour. Technol. 101, 3097–3105. doi:10.1016/j.biortech.2009.12.026

Daufresne, T., Lacroix, G., Benhaim, D., Loreau, M., 2008. Coexistence of algae and bacteria: a test of the carbon hypothesis. Aquat. Microb. Ecol. 53, 323–332. doi:10.3354/ame01254

Deller, S., Mascher, F., Platzer, S., Reinthaler, F.F., Marth, E., 2006. Effect of solar radiation on survival of indicator bacteria in bathing waters. Cent. Eur. J. Public

Health 14, 133–7.

Díez, B., Pedrós-Alió, C., Marsh, T.L., Massana, R., 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. 67, 2942–51. doi:10.1128/AEM.67.7.2942-2951.2001

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–200. doi:10.1093/bioinformatics/btr381

Falås, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T.A., Joss, A., 2016. Tracing the limits of organic micropollutant removal in biological wastewater treatment. Water Res. 95, 240–249. doi:10.1016/j.watres.2016.03.009

Garcia-Rodríguez, A., Matamoros, V., Fontàs, C., Salvadó, V., 2014. The ability of biologically based wastewater treatment systems to remove emerging organic contaminants-a review. Environ. Sci. Pollut. Res. 21, 11708–11728. doi:10.1007/s11356-013-2448-5

Gonçalves, A.L., Pires, J.C.M., Simões, M., 2016. A review on the use of microalgal consortia for wastewater treatment. Algal Res. doi:10.1016/j.algal.2016.11.008

Hom-Diaz, A., Jaén-Gil, A., Bello-Laserna, I., Rodríguez-Mozaz, S., Vicent, T., Barceló, D., Blánquez, P., 2017. Performance of a microalgal photobioreactor treating toilet wastewater: Pharmaceutically active compound removal and biomass harvesting. Sci. Total Environ. 592, 1–11. doi:10.1016/j.scitotenv.2017.02.224

Hom-Diaz, A., Llorca, M., Rodríguez-Mozaz, S., Vicent, T., Barceló, D., Blánquez, P., 2015. Microalgae cultivation on wastewater digestate: β-estradiol and 17α-ethynylestradiol degradation and transformation products identification. J. Environ.

Manage. 155, 106–113. doi:10.1016/j.jenvman.2015.03.003

Jobling, S., Nolam, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread Sexual Disruption in Wild Fish. Environ. Sci. Technol. 32, 2498–2506. doi:10.1021/es9710870

Judd, S., van den Broeke, L.J.P., Shurair, M., Kuti, Y., Znad, H., 2015. Algal remediation of CO2 and nutrient discharges: A review. Water Res. 87, 356–366. doi:10.1016/J.WATRES.2015.08.021

Jürgens, M.D., Holthaus, K.I.E., Johnson, A.C., Smoth, J.J.L., Hetheridge, M., Williams, R.J., 2002. The potential for estradiol and ethinylestradiol degradation in English rivers. Environ. Toxicol. Chem. 21, 480–488.

Koreivienė, J., Valčiukas, R., Karosienė, J., Baltrėnas, P., 2014. Testing of *Chlorella/Scenedesmus* microalgae consortia for remediation of wastewater, CO_2 mitigation and algae biomass feasibility for lipid production. J. Environ. Eng. Landsc. Manag. 22, 105–114. doi:10.3846/16486897.2013.911182

Kumar, G., Bakonyi, P., Kobayashi, T., Xu, K., Sivagurunathan, P., Kim, S., Buitrón, G., Nemestóthy, N., Béla, K., 2016. Enhancement of biofuel production via microbial augmentation: The case of dark fermentative hydrogen. Renew. Sustain. Energy Rev. 57, 879–891.

Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. Biotransformation and bioconcentration of steroid estrogens by *Chlorella vulgaris*. Appl. Environ. Microbiol. 68, 859–864. doi:10.1128/AEM.68.2.859

Lee, C.S., Lee, S.-A., Ko, S.-R., Oh, H.-M., Ahn, C.-Y., 2015. Effects of photoperiod on nutrient removal, biomass production, and algal-bacterial population dynamics in lab-scale photobioreactors treating municipal wastewater. Water Res. 68, 680–691. doi:10.1016/J.WATRES.2014.10.029

Martinez, J., Smith, D.C., Steward, G.F., Azam, F., 1996. Variability in ectohydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea. Aquat. Microb. Ecol. 10, 223–230.

Nübel, U., Garcia-Pichel, F., Muyzer, G., 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl. Environ. Microbiol. 63, 3327–32.

Peng, F.-Q.Q., Ying, G.-G.G., Yang, B., Liu, S., Lai, H.-J.J., Liu, Y.-S.S., Chen, Z.-F.F., Zhou, G.-J.J., 2014. Biotransformation of progesterone and norgestrel by two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*): Transformation kinetics and products identification. Chemosphere 95, 581–588. doi:10.1016/j.chemosphere.2013.10.013

Ramanan, R., Kim, B.H., Cho, D.H., Oh, H.M., Kim, H.S., 2016. Algae-bacteria interactions: Evolution, ecology and emerging applications. Biotechnol. Adv. 34, 14–29. doi:10.1016/j.biotechadv.2015.12.003

Rasconi, S., Niquil, N., Sime-Ngando, T., 2012. Phytoplankton chytridiomycosis: community structure and infectivity of fungal parasites in aquatic ecosystems. Environ. Microbiol. 14, 2151–2170. doi:10.1111/j.1462-2920.2011.02690.x

Renuka, N., Sood, A., Ratha, S.K., Prasanna, R., Ahluwalia, A.S., 2013. Evaluation of microalgal consortia for treatment of primary treated sewage effluent and biomass production. J. Appl. Phycol. 25, 1529–1537. doi:10.1007/s10811-013-9982-x

Salama, E., Kurade, M.B., Abou-Shanab, R.A.I., El-dalatony, M.M., Yang, I.-S., Min, B., Jeon, B.-H., 2017. Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation. Renew. Sustain. Energy Rev. 79, 1189–1211. doi:10.1016/j.rser.2017.05.091

Santos, L.H.M.L.M., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Ecotoxicological aspects related to the presence

590 of pharmaceuticals in the aquatic environment. J. Hazard. Mater. 175, 45–95.
591 doi:10.1016/j.jhazmat.2009.10.100
592Servos, M.R., Bennie, D.T., Burnison, B.K., Jurkovic, A., McInnis, R., Neheli, T.,
593 Schnell, A., Seto, P., Smyth, S.A., Ternes, T.A., 2005. Distribution of estrogens,
594 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. Sci.
595 Total Environ. 336, 155–70. doi:10.1016/j.scitotenv.2004.05.025
596Shi, W., Wang, L., Rousseau, D.P.L., Lens, P.N.L., 2010. Removal of estrone, 17α-
597 ethinylestradiol, and 17β-estradiol in algae and duckweed-based wastewater
598 treatment systems. Environ. Sci. Pollut. Res. Int. 17, 824–33. doi:10.1007/s11356-
599 010-0301-7
600Sime-Ngando, T., 2012. Phytoplankton Chytridiomycosis: Fungal parasites of
601 phytoplankton and their imprints on the food web dynamics. Front Microbiol 3,
602 361. doi:10.3389/fmicb.2012.00361
603Uggetti, E., Sialve, B., Trably, E., Steyer, J.-P., 2014. Integrating microalgae production
604 with anaerobic digestion: a biorefinery approach. Biofuels, Bioprod. Biorefining 8,
605 516–529. doi:10.1002/bbb.1469
606Vader, J.S., Ginkel, C.G. Van, Sperling, F.M.G.M., Jong, J. De, Boer, W. De, Graaf, J.S.
607 De, Most, M. Van Der, Stokman, P.G.W., 2000. Degradation of ethinyl estradiol by
608 nitrifying activated sludge. Chemosphere 41, 1239–1243. doi:0045-6535/00
609Whitton, R., Le Mével, A., Pidou, M., Ometto, F., Villa, R., Jefferson, B., 2016.
610 Influence of microalgal N and P composition on wastewater nutrient remediation.
611 Water Res. 91, 371–378. doi:10.1016/J.WATRES.2015.12.054
612Zhao, Y., Hu, J., Jin, W., 2008. Transformation of oxidation products and reduction of
613 estrogenic activity of 17β-estradiol by a heterogeneous photo-Fenton reaction.
614 Environ. Sci. Technol. 42, 5277–84. doi:10.1021/es703253q

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