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Removal of estrogens from primary settled sewage by repeated culture of *Selenastrum capricornutum*

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ABSTRACT

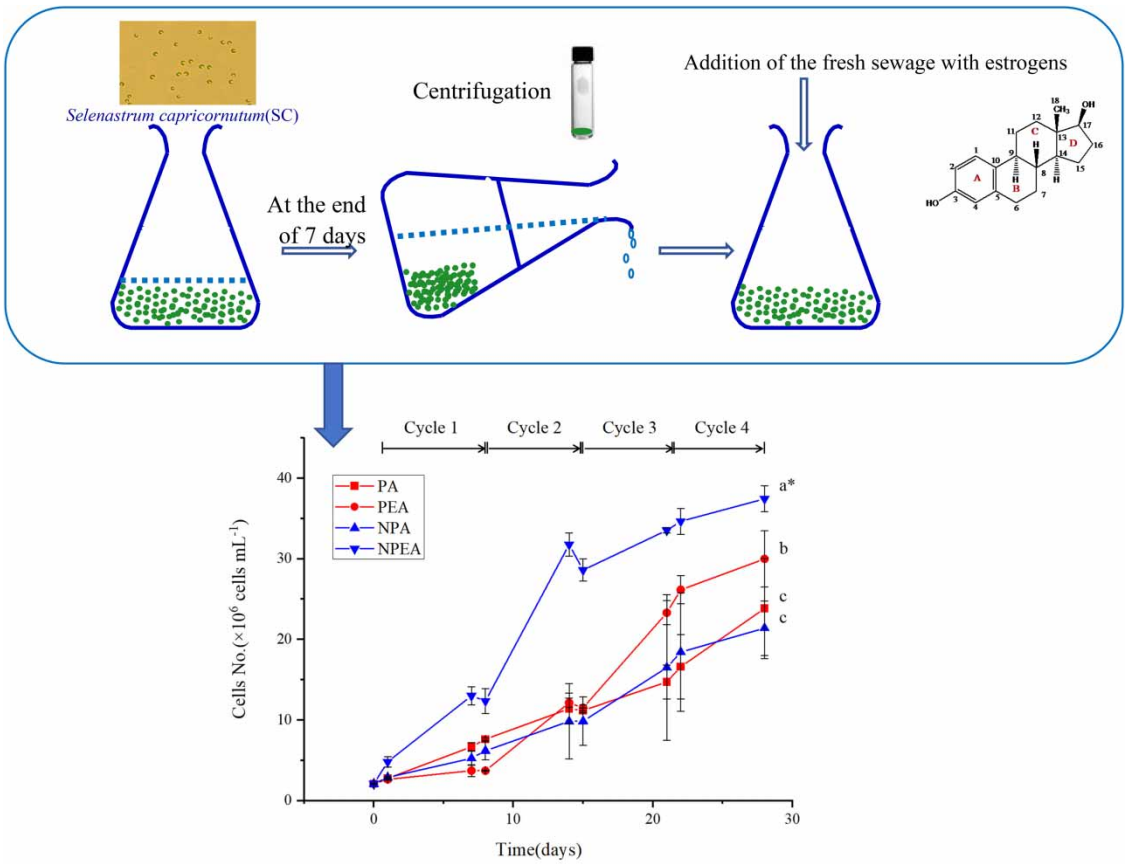
Biotransformation and biodegradation of estrogenic compounds by bacteria and even fungi have been reported widely, but the role of microalgae in the elimination of estrogens from municipal wastewater treatment plants and their interaction with other microorganisms in wastewater are not clear. This study reported the feasibility of repeatedly removing a mixture of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), each was 100 μg L^{-1}, from primary settled municipal sewage by *Selenastrum capricornutum* (SC), a ubiquitous microalga, in four exposure cycles, each lasted 7 days, and how they interacted with the microbial consortium in sewage. Mixed estrogen in sewage stimulated the growth of SC, and the indigenous microorganisms in sewage also affected the microalgal growth. The indigenous microorganisms, particularly bacteria, could easily remove E2 (with 99.5% removal), so the role of SC was insignificant. On the contrary, EE2 was difficult to remove by indigenous microorganisms but the removal was significantly enhanced by SC, with almost all spiked EE2 being removed, even at the end of the fourth cycle (with 99.0% removal). These results indicated that SC, together with the indigenous microorganisms in wastewater, could be repeatedly used for simultaneous removal of E2 and EE2 from municipal sewage.

Key words: biotransformation, estrogens, indigenous microbes, microalgae, wastewater

HIGHLIGHTS

- Growth of *S. capricornutum* (SC) was affected by estrogen and sterilization.
- The role of SC to remove E2 was insignificant as indigenous microbes were effective.
- The inoculation of SC significantly enhanced the removal of EE2 in sewage.
- SC could be repeatedly used to remove synthetic EE2 from sewage.

GRAPHICAL ABSTRACT



INTRODUCTION

Endocrine-disrupting chemicals, especially the most potent ones, such as estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), have been found in the influent and effluent of wastewater treatment plants (WWTPs). The concentrations of E1, E2 and EE2 in the influent of global WWTPs varied from 7.3 to 197 ng L^{-1}, 4.9 to 48 ng L^{-1} and <0.2 to 7,890 ng L^{-1}, respectively (Xu *et al.* 2012; Tang *et al.* 2021). Adeyeye & Laub (2020) reported that the average concentrations of WWTP effluent (E2, 41.43 $\pm$ 15.48; EE2, 11.40 $\pm$ 2.07 ng L^{-1}) were higher compared with the concentrations in Cibolo Creek, both downstream (E2, 30.09 $\pm$ 25.85; EE2, 6.33 $\pm$ 1.92 ng L^{-1}) and upstream (E2, 12.91 $\pm$ 11.12; EE2, 4.5 $\pm$ 1.38 ng L^{-1}) of WWTP inputs. Several abnormally high concentrations of EE2, in the range of 133.1–622.9 ng L^{-1}, were also reported in WWTPs in China (Xu *et al.* 2014; Fang *et al.* 2019). Fang *et al.* (2019) reported only low to moderate aqueous phase removals for E2 and EE2 (<64%) in WWTP. Liu *et al.* (2015) found that estrogen removal by full-scale municipal WWTPs varied greatly, and the removal efficiencies of E1, E2 and estriol (E3) fluctuated from −477 to 100% (average 37.8%), 0 to 100% (average 75.9%) and −175% to 100% (average 74.8%), respectively. They found that the average removal rates of E1 were less and even negative removal efficiencies occurred with the biotransformation of other estrogens and their conjugates. Our previous studies also showed that E2 could be readily transformed to E1 (Wang *et al.* 2017). Layton *et al.* (2000) also showed that 70–80% of E2 was mineralized to carbon dioxide (CO_2) within 24 h by the biosolids in the treatment processes in a WWTP in Tennessee, USA, whereas the mineralization of EE2 was only 40%, indicating that EE2 was more difficult to remove. However, EE2 could be degraded completely within 6 days by the nitrifying activated sludge in WWTPs with the formation of hydrophilic compounds (Vader *et al.* 2000). The estrogen removal processes in WWTPs in China were multi-faceted, including cleavage of conjugates, sorption to activate sludge and biodegradation by activated sludge, and the last one appeared to be the primary elimination process (Xu *et al.* 2014).

The cooperation among different microorganisms in wastewater and sludge plays an important role in the removal of organic pollutants in WWTPs (Shchegolkova *et al.* 2016; Cao *et al.* 2021). Heterotrophic bacteria could utilize free oxygen for efficient bioremediation of organic compounds and produce carbon dioxide (CO_2), which could, in turn, be utilized by microalgae for photosynthesis; microalgae could then assimilate inorganic nutrients and release oxygen through the photosynthetic process (Samorì *et al.* 2013). Microalgae could also accumulate and degrade wastewater-borne organic pollutants, such as nonylphenol, polycyclic aromatic hydrocarbons, active pharmaceutical ingredients and other toxic contaminants (phenol, bisphenol, tributyltin, and so on) (Ke *et al.* 2010; Baldiris-Navarro *et al.* 2018; Lindberg *et al.* 2021). However, the role of microalgae in the elimination of estrogens from WWTPs is not clear, especially in the presence of other indigenous microorganisms, as previous studies mainly focused on the role of the inoculated microalgae without any other microorganisms. The wastewater used is often artificially prepared contaminated wastewater but not the real wastewater collected from WWTPs. It is obvious that real wastewater is more complicated in nature than the artificial one, and the real wastewater harbors many different microorganisms, including bacteria, fungi, microalgae and even macroalgae (Abdel-Raouf *et al.* 2012; Zhang *et al.* 2020).

The interactions between the inoculated microalgae and the other microorganisms present in wastewater during treatment processes seldom are studied. Limited research showed that the bacteria in sewage could either stimulate or inhibit algal growth (de-Bashan *et al.* 2002, 2004; Zhang *et al.* 2012). The bacterial–algal interactions, the growth rates of the inoculated microalgae and their efficiency in removing contaminants could be affected by the characteristics of sewage, which vary significantly among sewage types (Viitasalo *et al.* 1992; Pérez *et al.* 2005). Different types of sewages, having different nutrient compositions, light and turbidity level, had different effects on the growth of an inoculated microalga, *Cladophora glomerata* (Viitasalo *et al.* 1992). Pérez *et al.* (2005) found that the biodegradability of antimicrobials in four different sewage treatment stages depended heavily on the wastewater matrix. The presence of *Azospirillum brasilense* was found to increase the removal of ammonium and phosphorus by *Chlorella vulgaris* (CV) (de-Bashan *et al.* 2002). Warshawsky *et al.* (2007) reported that the degradation of benzo[a]pyrene was enhanced by the conjunction of *Mycobacterium* sp. with a green microalga, *Selenastrum capricornutum* UTEX 1648. The fate of endocrine disrupters also varied in the different stages of sewage treatment, and natural estrogens were largely degraded biologically in denitrifying and aerated nitrifying tanks, whereas EE2 was only degraded in the nitrifying tank of the activated sludge system (Limpiyakorn *et al.* 2011).

Most previous researchers using microalgae to remove pollutants were carried out in a single cycle and seldom repeatedly used the same culture for consecutive cycles. This is not effective in wastewater treatment, as new cultures need to be prepared. Live *Phanerochaete chrysosporium* could still efficiently remove phenanthrene after five repeated cycles (each 6-day of incubation) (Ding *et al.* 2013). However, the effect of repeated exposure of microalgae to wastewater contaminated with estrogens and the removal efficiency have not been reported. The present study aims to investigate the growth of SC in primary settled municipal sewage collected from a local WWTP spiked with estrogens in four repeated cycles of exposure, each lasting 7 days, and the removal of estrogens in these four cycles. The study also evaluates the removal and biotransformation of estrogens by SC in the presence of indigenous microorganisms in real sewage.

METHODS

Collection of wastewater samples

The primary settled sewage, after preliminary screening, de-gritting and primary sedimentation processes was collected from Yuen Long Municipal Wastewater Treatment Plant (WWTP) in Hong Kong. The primary settled sewage had a pH of 6.0 $\pm$ 0.7, salinity of 0.95 $\pm$ 0.10 parts per thousand, ammonium of 20.89 $\pm$ 0.62 mg L^{-1}, phosphate of 3.74 $\pm$ 0.11 mg L^{-1}, copper of 0.006 $\pm$ 0.0001 mg L^{-1}, nickel of 0.004 $\pm$ 0.001 mg L^{-1}, zinc of 0.004 $\pm$ 0.001 mg L^{-1} and manganese of 0.095 $\pm$ 0.003 mg L^{-1}, respectively. The sewage had 64.48 $\pm$ 7.47 ng L^{-1} E2 and 2.30 $\pm$ 0.82 ng L^{-1} EE2. All values were mean and standard deviation of triplicates. The other contaminants such as lead, cadmium and nitrate were not detected in the sewage sample at the detection limits of 0.2, 0.022 and 0.001 mg L^{-1}, respectively. The sewage samples were stored overnight for solid settling at 4 °C and then filtered using quality medium filter paper (11 cm diameter, 30–50 μm, Oriental Chemicals and Lab. Supplies Ltd, Hongkong, China) to remove suspended solids. The filtrate was used as the culture medium.

Microalgal species and culture condition

The removal and biotransformation of estrogens by six microalga species, including three local isolates of *Chlamydomonas* sp. (WW), *Chlorella* sp. (2f5aia) and *Chlorella* sp. (1uoai), and three commercially available species, namely

Scenedesmus quadricauda (SQ), SC and CV, were compared in our previous study (Wang *et al.* 2017). The biological removal of estrogen was species-dependent. Among six species, SC was the most effective species to remove estrogen, and achieved the highest removal percentages of both E2 and EE2 among all microalgae. The green microalga species of SC was selected in this study, which was recently renamed *Pseudokirchneriella subcapitata* (Korshikov) Hindak, purchased from Carolina Biological Supply Company, Burlington, VT, USA. It was grown in Bristol medium (James 1978). The culture system was described in our previous work (Wang *et al.* 2017), and the harvested microalgal cells were washed and re-suspended in wastewater.

Experimental set-up and analysis

Part of the settled sewage was sterilized by autoclaving at 121 °C for 20 min to inactive the indigenous microorganisms, including bacteria, fungi, and micro and macro algae in wastewater, and the other part was non-sterilized. The sterilized and non-sterilized groups were further divided into three treatments: (i) estrogen control: sewage was spiked with mixed E2 and EE2 (purity >98%, Sigma-Aldrich, St. Louis, MO, USA), each at 100 μg L^{-1}; (ii) microalgal control: stock culture of SC was inoculated in sewage without any estrogen spiked; and (iii) estrogen + microalgae: same as (ii) but sewage was spiked with mixed E2 and EE2 at the same concentration as (i). The estrogen control in sterilized sewage (PA) and non-sterilized sewage (NPA) represented abiotic removal and abiotic plus biotic removal by indigenous microorganisms, respectively. Each treatment was in triplicate. For each set-up, the 1,000-mL Erlenmeyer flask containing 600 mL of sewage was used as the reactor and the initial biomass of SC was 70 mg L^{-1}.

The Erlenmeyer flasks were shaken on a rotary shaker under the same culture condition as described in our previous work for 7 days (40 μmol photons s^{-1} m^{-2} at 23 ± 1 °C, and a diurnal cycle of 16 h light and 8 h dark) (Wang *et al.* 2017) and this was the first cycle. At Days 1 and 7, 500 μL of the sample were harvested for counting cell numbers with a Nuebauer counting chamber under a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany) at a magnification of 400×. Another 15 mL of sample was collected for the analysis of residual estrogen in sewage and the uptake of estrogen by cells. At the end of the first cycle, microalgal cells were separated from the treated sewage by centrifugation (5,000 *g* for 15 min at 4 °C) and the cell pellets were re-suspended in another batch of freshly sewage to start the second cycle. This procedure was repeated for four cycles, each lasted 7 days with the same sampling times as described in the first cycle.

Extraction and analysis of estrogens in sewage and microalgal cells

The extraction and analysis of estrogens in sewage and microalgal cells were described in our previous paper (Wang *et al.* 2017). After sampling, estrogens in the sewage were extracted twice with ethyl acetate (25 mL) by liquid–liquid extraction, and the estrogens uptake in cell pellets were extracted with ethyl acetate (4 mL) mixed with 1 mL of 6 mol L^{-1} sodium hydroxide. 1 μg of 4-n-nonylphenol (4-n-NP) at a final concentration of 1 mg L^{-1} as the internal surrogate standard was used to determine the final recovery (Wang *et al.* 2017). The twice extracts were combined, evaporated, derivatized (70 °C, 1 h) and analyzed by gas chromatography-mass spectrometry (GC-MS).

Quality control was carried out to ensure the recovery efficiencies of estrogen extraction before the experiment. The recoveries for liquid–liquid extraction for the aqueous supernatant and the algal cells ranged from 80.4 to 92.8% for different compounds.

Statistical analysis

A parametric two-way analysis of variance (ANOVA) was used to test any significant difference in cell number among treatments and exposure cycles. The same test was used to test any significant effects between sterilization and estrogen effect in each cycle. Tukey's multiple comparisons were applied as the post hoc test to determine where the differences occurred when ANOVA results were significant at $p \leq 0.05$. If the interaction between these two sources of variations was significant, a one-way ANOVA was used to test the treatment effect in each cycle. All statistical analyses were carried out by a PC-compatible software package called SPSS (Version 16.0, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Growth of SC in four repeated exposure cycles

The cell number continuously in four repeated cycles and 7-day growth patterns in each cycle was comparable but patterns and rates varied among treatments (Figure 1). Growth was fastest in non-sterilized sewage spiked with estrogen (NPEA),

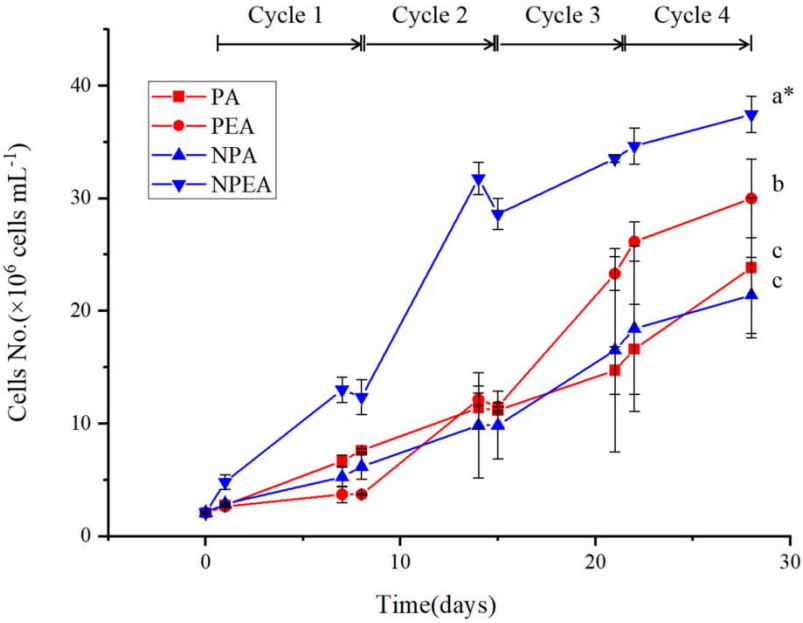


Figure 1 | Cell number of SC cultivated in primary settled sewage under different treatments during four repeated culture cycles, mean and standard deviations of triplicates are shown; PA: SC in sterilized sewage (no estrogens and no indigenous microbes); PEA: PA + estrogens; NPA: SC in non-sterilized sewage (no estrogens but with indigenous microbes); NPEA: PEA + estrogens; * indicates the different treatments with the same letter at the end of the graph indicate they were not significantly different among four treatment at $P \leq 0.05$ according to two-way ANOVA results with the treatments and culture cycle as two factors.

followed by the treatment of sterilized sewage spiked with estrogen (PEA), and the two treatments without estrogen (the algal control in PA and NPA) had significantly lower growth rates (exposure cycle $P < 0.001$ for treatments and cycles according to the two-way ANOVA test). These results indicated that the estrogen spiked into sewage stimulated the microalgal growth, and the presence of indigenous microorganisms enhanced the stimulatory effect of estrogens, i.e., estrogen and sterilization had a synergistic effect on the growth of SC. In the microalgal control, without estrogens, there was no significant difference in cell numbers between NPA and PA controls, suggesting that there was no interference or competition of indigenous microorganisms on the growth of SC in primary settled, non-estrogen spiked sewage. The growth rates of SC in sewage without estrogen, with and without indigenous microorganisms (NPA and PA treatments, respectively), varied from 0.08 to 0.17 day^{-1} in four cycles, significantly lower than that with estrogens (Table 1). The study showed that SC could be repeatedly exposed and cultured in estrogen-contaminated primary settled sewage with indigenous microorganisms.

Wastewater contains valuable nutrients needed by microalgae for their growth, and microalgae grow in wastewater without the external addition of nutrients (Caporgno *et al.* 2015; Gomez-Serrano *et al.* 2015). The present study also showed that the cell numbers of the inoculated SC increased continuously in all wastewater treatments in the presence of indigenous microorganisms and estrogens, over the course of four repeated exposure cycles, a total of 28 days of culture. The presence of indigenous microorganisms, particularly bacteria, together with estrogens, in the non-sterilized, spiked primary settled sewage even stimulated the growth of SC, as reflected by the higher cell numbers than the control (without estrogens) or in PA. Ryu *et al.* (2014) also found that the growth of *C. vulgaris* in untreated wastewater was the greatest compared with that in filtered and sterilized wastewater, suggesting that certain wastewater components, such as bacteria, promote microalgal growth. Similarly, the microalgae-growth-promoting bacterium, *Azospirillum brasilense*, significantly stimulated the growth of freshwater microalgae, *C. vulgaris* and *Chlorella sorokiniana*, used for wastewater treatment (de-Bashan *et al.* 2002). Mouget *et al.* (1995) found that two obligate aerobic bacteria isolated form laboratory algal cultures, namely *Pseudomonas diminuta* and *Pseudomonas vesicularis*, could stimulate the growth of two green microalgae, *Scenedesmus bicellularis* and *Chlorella* sp., without releasing any growth-promoting substance. However, there were studies reporting that bacteria could inhibit the growth of microalgae in aquatic cultures. For instance, indigenous bacteria in unsterilized wastewater inhibited the growth of CV at >231 mg L^{-1} dissolved organic carbon (He *et al.* 2013). Zhang *et al.* (2012) found that although the bacteria that coexisted with the microalgae did not significantly affect the productivity of microalgae,

Table 1 | Specific growth rates [R (day^{-1})] of SC cultivated in primary settled sewage under different treatments during four repeated culture cycles, and two-way ANOVA results showing the effects of different treatments and cycles on the growth rates in each cycle

		Culture cycles			
Treatments		**1**	**2**	**3**	**4**
Sterilized	PA	0.17 ± 0.01^{a}	0.12 ± 0.01^{a}	0.09 ± 0.01^{a}	0.09 ± 0.01^{NS}
	PEA	0.08 ± 0.03^{b}	0.12 ± 0.03^{a}	0.11 ± 0.02^{a}	0.10 ± 0.00^{NS}
Non-sterilized	NPA	0.13 ± 0.02^{a}	0.11 ± 0.00^{a}	0.10 ± 0.00^{a}	0.08 ± 0.01^{NS}
	NPEA	0.26 ± 0.01^{c}	0.19 ± 0.00^{b}	0.13 ± 0.00^{b}	0.10 ± 0.00^{NS}
Two-way ANOVA results (F values)					
Treatments		33.9***			
Cycles		25.6***			
Interaction (treatments x cycles)		11.1***			

$R = Ln\ (N_2/N_1)/(T_2–T_1)$, where N_1 and N_2 = cell numbers at time T_1 and time T_2, respectively.
*** indicates $P \leq 0.001$ levels; NS: not significant; different letters in the superscript position of the mean ± SD in the same column indicate significant differences among treatments in each cycle at $p \leq 0.05$ according to one-way ANOVA.

in terms of biomass, the total lipid content and lipid production rate of microalgae reduced slightly. *Pseudomonas vesicularis* coexisted with several microalgal species could produce anti-algal substances to inhibit the growth of microalgae (Dakhama *et al.* 1993). The interactions between microalgae, particularly the inoculated ones, and the indigenous microorganisms in wastewater are complicated and deserve more in-depth studies.

Removal of E2 from sewage

The removal of E2 from primary settled sewage was efficient and achieved more than 98% in all treatments and all cycles during the study except in PA on Day 1 of the first cycle (Table 2). The PA with microalgal inoculation but without indigenous microorganisms only had 77% removal on Day 1 of the first cycle, while the respective removal in NPA was close to 100%. The removal was very rapid and occurred within the first day of exposure, and the same high removal percentages were maintained in the four repeated cycles (Table 2). In PA without indigenous microorganisms, SC could remove almost all spiked E2, except on Day 1 of the first cycle (Table 2). On the contrary, the removal was mainly due to the transformation of estrogen

Table 2 | Mean removal percentage of E2 in different algal-wastewater treatment systems during four repeated cycles of culture, standard deviations of triplicates are shown

	Days	
Cycle	**Day 1**	**Day 7**
Sterilized		
1	76.8 ± 4.2	99.6 ± 0.0
2	99.5 ± 0.2	99.4 ± 0.6
3	98.2 ± 2.5	99.9 ± 0.1
4	99.8 ± 0.2	99.8 ± 0.1
Abiotic removal in Cycle 1	7.6 ± 1.8	20.7 ± 1.6
Non-sterilized		
1	99.9 ± 0.0	99.9 ± 0.0
2	99.0 ± 0.3	99.7 ± 0.1
3	98.8 ± 0.3	99.9 ± 0.1
4	99.3 ± 0.3	100.0 ± 0.0
Abiotic and microbial removal in Cycle 1	96.3 ± 0.4	99.5 ± 0.1

% = (initial input – residual amount of estrogen in treatment)/initial input × 100.

(E2) by indigenous microorganisms in NPA, and the inoculation of SC had little role in such removal (less than 3.6% of the spiked E2 was removed by SC). The abiotic and biotic removal by indigenous microbes, without microalgal inoculation, achieved 96 and 99% removal in Day 1 and 7, respectively. These results suggested that the indigenous microorganisms in sewage were effective in removing E2 as SC.

The mass balance results of E2 showed that the amounts that remained in water fraction (medium) and accumulated in biomass were very low in all samples, especially at Day 7, and the values were comparable among four cycles (Table 3). The amounts of E2 uptake in biomass were relatively higher at Day 1 than 7 in third and fourth cycles, and the amounts of E2 accumulated in biomass on Day 7 were more or less the same among the four cycles. There was also no significant difference in cellular uptake of E2 between sterilized and NPAs, suggesting that the uptake of E2 by SC was similar to that by indigenous microorganisms. The unrecovered amount (around 60 μg E2) accounted for almost 100% of the initial input at the end of each cycle in both sterilized and NPAs. These findings indicated exposure cycles that E2 in the cell biomass or in water might be biotransformed by SC and/or the indigenous microorganisms in sewage to estrone (E1) and other products.

Removal of EE2 from sewage

It was more difficult to remove EE2 than E2 in sewage and the removal percentages of EE2 at Day 1 were significantly lower than that at Day 7 in all treatments (Table 4). In each cycle, EE2 removal reached 80–100% at Day 7. The removal at Day 1 increased with the number of cycles and reached 48% in the fourth cycle in the PA with the inoculation of SC but without indigenous microorganisms. There were no significant differences in the removal of EE2 between sterilized and NPAs during the four cycles ($P = 0.68$ according to two-way ANOVA), indicating that the microalgal removal ability was not interfered with by the presence of indigenous microorganisms in sewage. The abiotic loss of EE2 in PA was less than 5%, while the removal in NPA with indigenous microorganisms but without microalgal inoculation could be as high as 35.4% on Day 7 (Table 4). Very different from E2 removal, the role of SC in the removal of EE2 in both sterilized and NPAs was very important and accounted for more than 64.6% of EE2 removal, while indigenous microorganisms contributed less than 30%.

Table 3 | Mass balance of E2 in sterilized and non-sterilized primary settled sewage inoculated with SC during four repeated cycles, mean and standard deviations of three replicates are shown

Amount of E2 (μg)		Sterilized (just SC, no indigenous microbes)		Non-sterilized (SC together with indigenous microbes)	
Cycle		Day 1	Day 7	Day 1	Day 7
1	*Medium*	14.07 ± 2.56	0.24 ± 0.03	0.08 ± 0.02	0.04 ± 0.01
	Biomass	0.05 ± 0.01	0.03 ± 0.00	0.01 ± 0.00	0.05 ± 0.07
	Sum	14.13 ± 2.54	0.27 ± 0.03	0.10 ± 0.03	0.09 ± 0.08
	Unrecovered	46.47 ± 2.54	60.32 ± 0.03	60.50 ± 0.03	60.51 ± 0.08
2	*Medium*	0.30 ± 0.14	0.38 ± 0.37	0.58 ± 0.19	0.19 ± 0.04
	Biomass	0.04 ± 0.00	0.09 ± 0.01	0.43 ± 0.12	0.09 ± 0.06
	Sum	0.35 ± 0.14	0.48 ± 0.37	1.01 ± 0.31	0.28 ± 0.09
	Unrecovered	60.25 ± 0.14	60.12 ± 0.37	59.59 ± 0.31	60.32 ± 0.09
3	*Medium*	1.10 ± 1.54	0.08 ± 0.05	0.73 ± 0.19	0.08 ± 0.08
	Biomass	1.39 ± 0.40	0.14 ± 0.07	0.56 ± 0.12	0.02 ± 0.00
	Sum	2.49 ± 1.93	0.22 ± 0.02	1.29 ± 0.31	0.10 ± 0.08
	Unrecovered	58.10 ± 1.93	60.38 ± 0.02	59.31 ± 0.31	60.49 ± 0.08
4	*Medium*	0.13 ± 0.13	0.10 ± 0.09	0.44 ± 0.19	0.01 ± 0.00
	Biomass	1.35 ± 0.16	0.10 ± 0.02	0.67 ± 0.17	0.02 ± 0.01
	Sum	1.48 ± 0.07	0.20 ± 0.08	1.12 ± 0.02	0.03 ± 0.01
	Unrecovered	59.11 ± 0.07	60.39 ± 0.08	59.48 ± 0.02	60.56 ± 0.01

Initial input for each cycle was 60.59 ± 4.76 μg.

Table 4 | Mean removal percentage of EE2 in different algal-wastewater treatment systems during four repeated cycles of culture, standard deviations of three replicates are shown

Removal of EE2	Time	
Cycle	Day 1	Day 7
Sterilized		
1	24.3 ± 2.1	81.6 ± 10.6
2	32.4 ± 6.3	89.4 ± 3.2
3	38.9 ± 3.9	98.1 ± 0.9
4	48.4 ± 3.6	99.2 ± 0.2
Abiotic removal in Cycle 1	2.5 ± 1.3	21.7 ± 3.3
Non-sterilized		
1	15.8 ± 7.3	79.1 ± 1.1
2	38.2 ± 3.8	96.2 ± 1.8
3	45.2 ± 4.1	98.4 ± 0.9
4	33.8 ± 3.8	99.0 ± 0.3
Abiotic and microbial removal in Cycle 1	1.1 ± 0.5	35.4 ± 1.7

% = (initial input – residual amount of estrogen in treatment)/initial input × 100.

The mass balance calculation showed that the amounts of EE2 that remained in the medium were very high on Day 1 but dropped significantly on Day 7 of each cycle (Table 5). This suggested that EE2 was much more stable than the estrogen (E2) in sewage. On the contrary, the unrecovered amounts of EE2 increased from Days 1 to 7 of each cycle. Similarly, the unrecovered amounts in the fourth cycle were also higher than that in the first cycle. These results indicated that EE2 could

Table 5 | Mass balance of EE2 in algal-wastewater system of species of SC exposed to sterilized and non-sterilized primary settled sewage during four repeated cycles

Amount of EE2/μg		Sterilized (just SC, no indigenous microbes)		Non-sterilized (SC together with indigenous microbes)	
Cycle		Day 1	Day 7	Day 1	Day 7
1	*Medium*	44.9 ± 1.2	10.9 ± 6.3	50.0 ± 4.3	12.4 ± 0.6
	Biomass	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
	Sum	45.3 ± 1.2	11.2 ± 6.3	50.2 ± 4.4	12.8 ± 0.7
	Unrecovered	14.0 ± 1.2	48.2 ± 6.3	9.1 ± 4.4	46.6 ± 0.7
2	*Medium*	40.1 ± 3.7	6.3 ± 1.9	36.7 ± 2.2	2.2 ± 1.1
	Biomass	0.7 ± 0.0	0.5 ± 0.1	1.7 ± 0.2	0.2 ± 0.1
	Sum	40.8 ± 3.7	6.8 ± 2.0	38.4 ± 2.4	2.4 ± 1.2
	Unrecovered	18.5 ± 3.7	52.6 ± 2.0	21.0 ± 2.4	56.9 ± 1.2
3	*Medium*	36.3 ± 2.3	1.1 ± 0.5	32.5 ± 2.4	1.0 ± 0.5
	Biomass	5.1 ± 1.1	0.4 ± 0.2	2.0 ± 0.3	0.3 ± 0.1
	Sum	41.4 ± 1.3	1.6 ± 0.7	34.5 ± 2.3	1.3 ± 0.6
	Unrecovered	18.0 ± 1.3	57.8 ± 0.7	24.9 ± 2.3	58.1 ± 0.6
4	*Medium*	30.6 ± 2.1	0.5 ± 0.1	39.3 ± 2.3	0.6 ± 0.2
	Biomass	4.2 ± 0.2	0.4 ± 0.1	2.2 ± 0.6	0.3 ± 0.1
	Sum	34.8 ± 2.3	0.9 ± 0.2	41.5 ± 2.8	0.9 ± 0.3
	Unrecovered	24.6 ± 2.3	58.5 ± 0.2	17.8 ± 2.8	58.4 ± 0.3

Initial input for each cycle was 59.4 ± 7.0 μg.

gradually be biotransformed by SC and other indigenous microorganisms in sewage with time. The amounts of EE2 uptake in cell biomass were relatively small in all treatments, suggesting that SC and indigenous microorganisms bioaccumulated little EE2.

Comparision of E2 and EE2 removal efficiencies

Both natural and synthetic estrogens share the same tetracyclic molecular framework and the only difference in the compound lies in the configuration of the cyclopentane ring at positions C16 and C17 (Khanal *et al.* 2006) (Supplementary Figure S1). For natural estrogens, estrone has a carbonyl group on C17, estradiol has a hydroxyl group on C17 and estriol has two hydroxyl groups on C16 and C17. For the synthetic estrogens, ethinylestradiol has an ethynyl group on C17, and estradiol valerate has a valeryl group on C17 (Fang *et al.* 2001). The D-ring generally gets attacked firstly in biotransformation and the A-ring firstly cleaves by photolysis (Lee & Liu 2001). Yi & Harper (2007) also showed that the A-ring was the most sensitive site for electrophilic reactions because of its high electron density.

The removal efficiencies of estrogens in WWTPs in China varied widely; 10–100% for E1, 39.6–100% for E2, and 0–>98% for EE2, with respective average percentages of 78, 89 and 74% (Xu *et al.* 2014). The same study also reported that the biodegradation half-lives of estrogens in an activated sludge treatment process were significantly longer for EE2 (from 0.47 h to several days), followed by E1 (from 0.15 to 24 h) and E2 had the shortest half-lives (from 0.05 to 15 h). This suggests that WWTPs employing activated sludge processes were less effective in removing E1 and EE2 than E2, and the removal percentages of the first two estrogens fluctuated. Other studies also showed that E1 and EE2 were more persistent than the other estrogens in WWTPs (Liu *et al.* 2015; Fang *et al.* 2019). Wang *et al.* (2010) found that EE2 was very persistent and hardly affected by the activated sludge in WWTP, while E2 could easily be oxidized and further eliminated under the same conditions. Further, the EE2 removal in a WWTP in England employing the nitrifying activated sludge treatment process was poor, with only 3% for 24 h and 5.6% for 7 days (Kanda & Churchley 2008). In this study, the removal of estrogen E2, by just the indigenous microorganisms in sewage or by the inoculated SC, was also very fast, but the removal of EE2 especially by the indigenous microorganisms alone, was significantly slower than E2. The importance of SC in the removal of EE2 was demonstrated in this study. Della Greca *et al.* (2008) also found that among 11 different microalgal strains, SC, SQ, *Scenedesmus vacuolatus* and *Ankistrodesmus braunii* could transform EE2 but the transformation processes and products were species-dependent.

The indigenous microorganisms, in particular, bacteria in wastewater or in activated sludge, played an important role in the removal of estrogens in WWTPs (Xu *et al.* 2014; Liu *et al.* 2015). Shi *et al.* (2004) found that ammonia-oxidizing bacteria (AOB) and other microorganisms in nitrifying active sludge were involved in the biodegradation of estrogens. The same study reported that an isolated strain, *Nitrosomonas europaea*, was able to decompose 1 mg L^{-1} of EE2 within 96 h during a batch experiment. The presence of an ammonium monooxygenase in AOB was important in the transformation of EE2 with the formation of hydroxylated metabolite (EE2-OH) (Yi & Harper 2007). The strains of *Rhodococcus zopfii* and *Rhodococcus equi* isolated from the activated sludge in WWTPs in Japan could degrade E1, E2, E3 and EE2, and *R. zopfii* Y50158 had the strongest degradation efficiency and removed 100 mg L^{-1} of EE2 within 24 h (Yoshimoto *et al.* 2004). Ren *et al.* (2007) also isolated an EE2-degrading bacterium, *Sphingobacterium* sp. JCR5, from the activated sludge in WWPTs in China, could utilize EE2 as the sole source of carbon and energy and metabolize up to 87% of the added EE2 at an initial concentration of 30 mg L^{-1} within 10 days. The present study also demonstrated the importance of microalgae SC in the biotransformation of EE2. The biotransformation percentages ranged from 78 to 98% in Cycles 1 to 4 after 7-day of incubation (Table 5). Although the transformation efficiencies were not as good as bacteria according to the above-mentioned literature, microalgae play a very important role in the removal of environmental pollutants, which is the first producer in the ecosystem.

The repeated use of microalgal-medium systems for the removal of pollutants has been extensively reported (Tam *et al.* 1998, 2001; Esteves *et al.* 2000; Saeed & Iqbal 2006). However, the biotransformation and biodegradation of organic pollutants have not received sufficient attention. In the present study, the removal percentages of E2 and EE2 by SC and indigenous microorganisms were nearly 100% at the end of the fourth cycle, suggesting that SC could be used repeatedly for estrogen removal. The generally accepted transformation processes of estrogens by microalgae were oxidation, hydroxylation and glycosylation (Della Greca *et al.* 2008). Della Greca *et al.* (2008) found that three transformation products of EE2 were identified in SC treatment through hydroxylation and glycosylation processes. Our previous study also demonstrated that E2 could be oxidized to E1, then hydroxylated to E3, and further degraded to unknown products by SC (Wang *et al.*

Table 6 | Correlation coefficient matrix between cell number, percentages of removal and uptakes of E2 and EE2 in primary settled sewage inoculated with *S. capricornutum* after four repeated cycles of culture

Correlation	E2 removal %	EE2 removal %	E2 uptake %	EE2 uptake %
Cell number	0.38*	0.49***	0.12^{NS}	0.06^{NS}
E2 removal %		0.36*	0.07^{NS}	0.07^{NS}
EE2 removal %			−0.38*	−0.42**
E2 uptake %				0.98***

$n = 48$; NS, not significant.

*, ** and *** indicate r values significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

2017). The relative roles and mechanisms of the indigenous microorganisms in wastewater and their coexistence of the inoculated microalgae in the removal of estrogens need further investigation.

Correlations between growth of SC and estrogen removal/uptake

In primary settled sewage, positive correlations were found between cell numbers and the removal of estrogens, indicating the more biomass, the higher removal (Table 6). However, there were no significant correlations between estrogen uptake and cell numbers. The uptake of E2 was positively related to the uptake of EE2, suggesting that the uptake process of these two estrogens might be comparable and there was no competition. The uptake of E2 or EE2 was not related to E2 removal but was negatively related to EE2 removal.

CONCLUSIONS

This is the first study demonstrating that inoculated SC cells could adapt to the sewage contaminated with mixed estrogens at a level of 200 μg L^{-1} for four repeated exposure cycles. The presence of other indigenous microorganisms, particularly bacteria, stimulated the growth of SC in estrogen-contaminated sewage. During the four repeated exposure cycles, the removal percentages of E2 and EE2 in primary settled sewage with the inoculation of SC were positively correlated with the cell numbers. Indigenous microorganisms in wastewater removed E2, and the role of SC was not significant. However, the inoculation of SC significantly enhanced the removal of EE2, a more resistant synthetic estrogen, with almost all EE2 being removed even at the end of the fourth cycle. These results indicated that SC could be used repeatedly to remove synthetic EE2, as this microalgal species was resistant to continuous exposure to E2 and EE2, and the presence of the indigenous microorganisms in sewage had little interference with the inoculated microalgae.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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