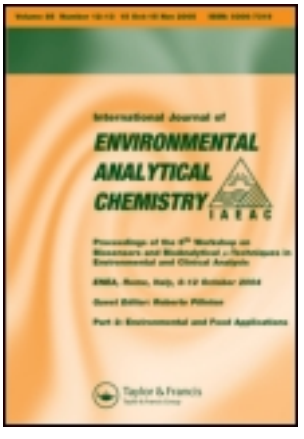


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# Elimination of estrogens and estrogenic activity from sewage treatment works effluents in subsurface and surface flow constructed wetlands

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The effluents discharged from sewage treatment works (STWs) are major sources of environmental estrogens, which poses an urgent need to explore appropriate techniques for effluent-polishing. In light of the debate concerning the effectiveness of constructed wetlands (CW) for the elimination of estrogens, the present study evaluated and compared the performance of two basic types of CW, free water surface (FWS) and subsurface flow (SSF) systems. Two FWS and two SSF field CW mesocosms were fed continuously with an STW effluent. All the mesocosms provided an effective elimination of estrogens and estrogenic activity. Unexpectedly, the performance of FWS mesocosms was not inferior to that of SSF mesocosms. Additional shading experiments demonstrated that the presence of filamentous green algae along with the sunlight enhanced the removal of estrogens and estrogenic activity in FWS mesocosms, enabling FWS mesocosms to perform comparably to SSF mesocosms. Microbial inhibition tests further indicated that *Spirogyra* sp. itself rather than algae-attached bacteria played an important role in the removal of estrogen and estrogenic activity.

**Keywords:** constructed wetland; estrogen; free water surface flow; subsurface flow; filamentous green algae



## 1. Introduction

Estrogens have become recognised as potential aquatic environmental contaminants during the past decades because of their adverse impacts on a wide variety of aquatic organisms, such as developmental and reproductive abnormalities in fish [1]. There are growing data on the presence of estrogens in surface freshwater [2], ground water [3], sea water [4] and sediments of river beds [5,6].

The principal estrogens of environmental concern are naturally occurring estrogens 17$\beta$-estradiol (E2) and estrone (E1), as well as a synthetic estrogen 17$\alpha$-ethinylestradiol (EE2) widely used for birth control and estrogen replacement therapy. Their estrogenic potency is several orders of magnitude higher than any other endocrine disrupting compounds [7,8]. Moreover, they are able to cause reproductive disturbances even

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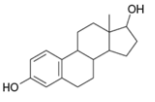
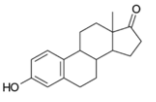
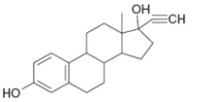
Table 1. Physical-chemical properties of 17$\beta$-estradiol (E2), estrone (E1) and 17$\alpha$-ethinylestradiol (EE2) [9–11].

| Common name | 17$\beta$-estradiol | estrone | 17$\alpha$-ethinylestradiol |
|---|---|---|---|
| IUPAC name | (17$\beta$)-estra-1,3,5(10)-triene-3,17-diol | 3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren-17-one | (8S,9S,13S,14S,17S)-17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol<br>19-nor-17$\alpha$-pregna-1,3,5(10)-trien-20-yne-3,17-diol |
| CAS number | 50-28-2 | 53-16-7 | 57-63-6 |
| Formula | $C_{18}H_{24}O_2$ | $C_{18}H_{22}O_2$ | $C_{20}H_{24}O_2$ |
| Structure |  |  |  |
| Molecular weight (g/mol) | 272.38 | 270.37 | 296.40 |
| Melting point (°C) | 171 | 259 | 183 |
| Ionisation constant (pKa) | 10.23 | 10.34 | 10.05 |
| Water solubility (mg/L) (25°C) | 3.9–13.3 | 0.8–12.4 | 4.8–19.1 |
| Vapour pressure (Pa) (25°C) | 3.0E–08 | 3.0E–08 | 6.0E–09 |
| Henry's law constant (atm $m^3$/mol) (25°C) | 3.64E–11 | 3.80E–10 | 7.94E–12 |
| Log $K_{OW}$ | 3.1–4.0 | 3.1–3.4 | 3.6–4.1 |



at concentrations as low as sub-nanogram per litre [6,7]. The physicochemical properties of E2, E1 and EE2 are given in Table 1. All of the three estrogens are regularly excreted in urine from the human body. Reported average human excretion of estrogens per person is approximately 10.5 and 6.6 μg/day for E1 and E2, respectively, while the population-normalised concentration of EE2 is 1.0 μg/day per person [8].

Human-derived estrogens commonly enter sewage treatment works (STW) via the sewage systems. Unfortunately, numerous field studies have suggested that estrogens were not totally eliminated during the biological wastewater treatment processes in STW though the concentrations of them were reduced substantially [1,4,7,8]. Hence, the discharge of STW effluents has been considered as the most significant entry route for estrogens into aquatic environments because the remaining estrogens in effluents are usually at concentrations higher than the maximum limit levels reported as producing estrogenic effects in fish and other aquatic organisms [1,12].

Given the strong link between estrogens and endocrine disruption in marine and freshwater fish, it is essential to alleviate their loads into the environment in spite of the lack of specific regulations concerning estrogen residues in the environment. To this end,

optimisation of biological wastewater treatment processes has been attempted, e.g. by increasing hydraulic retention time, sludge retention time, and dissolved oxygen concentration, but the complete removal of the estrogens is still a concern [12]. Therefore, there is a need to consider alternative strategies for the removal of trace amounts of estrogens present in STW effluents prior to their discharge into the aquatic environment. Some advanced treatment techniques such as advanced oxidative processes, activated carbon adsorption, membrane separation [12], photocatalysis processes [13] and ultrasound treatment [11], etc., have been successful on a small scale, but they are largely uneconomical and consequently unpractical at present for full scale treatment.

Constructed wetlands (CW) are man-made wetlands used to treat a variety of point and non-point source wastewaters and are emerging as a cost-saving environmentally friendly water treatment process. CW systems are increasingly being used to polish STW effluents, initially for reduction of nitrogen and phosphorus, and recently are also being used with success to remove some emergent pollutants of concern, such as pesticides [14] pharmaceutically active compounds, personal care products [15–18] and disinfection byproducts precursors' [19].

Nevertheless, researches on the behaviour of estrogens in CW have been very limited. One of the limited studies documented that the outflow of a CW in Texas had lower estrogenic activity than that of inflow [20]. On the contrary, data from an effluent-receiving CW indicated that estrogenic activity was not attenuated when water passed through the CW [21]. Apparently, there are controversial results in the literature, suggesting that further investigation is necessary.

Moreover, the two basic categories of CW are free water surface (FWS) and subsurface flow (SSF) systems, with the former maintaining a thin layer of water above the media whereas the water level of the latter system is kept just below the top of the permeable medium. In the literature, several studies have been conducted on the treatment of domestic wastewater using FWS and SSF systems [22]. However, comparison of both systems with regard to estrogens and estrogenic activity removal using identical filter media and macrophytes receiving the same polluted water under the same environmental conditions is not available.

Thus, the specific objectives of this study were to (1) determine if CWs are able to simultaneously reduce estrogens and estrogenic activity in STW effluents; (2) ascertain if SSF wetlands perform better than FWS wetlands with regard to the elimination of estrogens and estrogenic activity; (3) determine if filamentous green algae (*Spirogyra* sp.) observed in FWS wetlands are involved in the reduction of estrogens.



## 2. Experimental

### 2.1 *CW mesocosms*

Four CW mesocosms were set up at Kamiyagari STW in Sendai City, Japan. Two were SSF and the other two were FWS systems. Reported results are the average of duplicate mesocosms for SSF or FWS mesocosms. Each mesocosm was made of high-density polyethylene (HDPE) with the dimensions of 0.5 m in length, 0.3 m in width and 0.9 m in height. All the CWs were filled with sand (median grain diameter, $d_{50}$ of 1 mm, $d_{10}$ of 0.8 mm, $d_{90}$ of 1.2 mm, and a calculated porosity of 26%) to a depth of 0.6 m. A water depth of 0.5 m (i.e. 0.1 m below the sand surface) was maintained in the SSF mesocosms while 0.2 m above the sand surface in the FWS mesocosms. All the mesocosms were

planted with common reed (*Phragmites australis*) at an initial density of 60 seedlings/$m^2$. The CW mesocosms were fed with the effluent from Kamiyagari STW at a hydraulic loading rate (HLR) of 0.15 $m^3/(m^2 \cdot d)$. Before the start of the present study, the mesocosms had been run continuously for about one year.

### 2.2 *Field experiment design*

Water samples (grab samples) of inflow and outflow of all four CW mesocosms were collected in duplicate using 3.4 L silanised amber glass bottles with Teflon lined caps, previously detergent washed, acid rinsed, and pyrolysed at 400°C for 4 h in a muffle furnace. All samples were placed on ice during transport and stored at 4°C until extraction was performed, no later than 24 h after collection. Five sampling events were conducted in June and July for all the four CW mesocosms.

It should be pointed out that, during this period, we observed a noticeable growth of filamentous green algae (predominantly *Spirogyra* sp.) on the surface of the both FWS mesocosms. For the purpose of evaluating the effect of filamentous green algae on the attenuation of estrogens and estrogenic activity, a shading experiment was carried out during the following experiment period using the two FWS CW mesocosms. Since August, one of the two FWS CW mesocosms was covered several strips of black plastic sheet, in which the filamentous green algae had been cleared away beforehand. Another FWS CW mesocosm was left unchanged. During this period, there were four sampling events carried out from September to October.

### 2.3 *Reagents and chemicals*

Estrone (min. 98%), 17$\beta$-estradiol (97–103%) and 17$\alpha$-ethynylestradiol (min. 98%) were purchased from Wako Pure Chemicals (Tokyo, Japan). All the solvents, methanol (MeOH), ethyl acetate, acetonitrile (ACN), dichloromethane (DCM), dimethylsulfoxide (DMSO), acetone, and *n*-hexane, used in this study were of high performance liquid chromatography (HPLC) grade or residual pesticide grade purchased from Wako Pure Chemical Industries (Tokyo, Japan). Water was purified using Milli-Q system (Nihon Millipore, Tokyo, Japan). All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

### 2.4 *Sample preparation*

The sample preparation procedure was in accordance with the Japanese *Standard Methods for Sewage Tests* (Japan Sewage Works Association, 2002). Before extraction, 1000 mL water sample was filtered through GF/C and GF/D 47 mm glass microfibre filters (Whatman, Maidstone, Kent, UK) with a glass vacuum filtration device. The filtrate was extracted by Bond Elut® C18 solid-phase column (Varian, Harbor City, CA, USA) using an automatic solid-phase extraction apparatus (AQUA Trace ASPE699, GL Sciences, Tokyo, Japan) with the protocol recommended by the manufacturer. A quantity of 6 mL of ethylacetate/methanol (5 : 1) was used to elute estrogens from each C18 solid-phase column. The extract was evaporated to dryness at room temperature under a gentle stream of nitrogen gas, re-suspended in 1 mL of *n*-hexane/DCM (1 : 1), subjected to a ultrasonic bath for 10 min and then passed through a Bond Elut® florisil solid phase cartridge

(Varian, Harbor City, CA, USA) which had been rinsed with 10 mL of *n*-hexane/DCM (1 : 1). The eluent was then re-extracted from the florisil cartridge by eluting with 6 mL of acetone/DCM (1 : 9) and subsequently purged under a gentle nitrogen stream again. Finally, the residue was re-dissolved in 200 μL MeOH just before being subject to HPLC/MS analysis or yeast two-hybrid assay.

### 2.5 *HPLC/MS analysis*

HPLC/MS analysis was carried out as described in literature [23]. A gradient elution from 30% to 90% ACN in HPLC grade water for 20 min at a flow rate of 200 μL/min was used as mobile phase of HPLC. Chromatographic separation was achieved on an XTerra® MS C18 column (3.5 μm, 2.1 × 100 mm, Waters Corporation, Dublin, Ireland) preceded by a guard column (XTerra® RP 18, 3.5 μm, Waters Corporation, Dublin, Ireland). MS detection was performed under the time-scheduled selected ion monitoring (SIM) conditions by using an electrospray interface operating in the negative ion mode. MS conditions were as follows: nebulising gas flow, 1.5 L/min; curved desolvation line (CDL) voltage, −25 V; CDL temperature, 250°C; probe voltage, −4.5 V; and detector gain, 1.6 kV. Nitrogen was used as nebulising and drying gas. Quantitative analysis was accomplished using the selected ion monitoring (SIM) mode by external calibration.

The recovery of estrogens was ≥93%, and the relative standard deviation (RSD) of the method was ≤14%. The limit of detection (LOD) was 0.05 ng/L and the limit of quantification (LOQ) was 0.15 ng/L for each estrogen. Variability of repeated injections of the same sample was ≤3%. The variability of duplicate samples was ≤9%.

### 2.6 *Yeast two-hybrid assay*

Yeast two-hybrid assay (YTA) was used to determine the estrogenic activity in the present study. YTA was performed following method introduced by Dr. F. Shiraishi and his co-workers [24,25]. Briefly, this assay system used yeast cells (*Saccharomyces cerevisiae* Y190) carrying a *β*-galactosidase reporter gene, into which an estrogen-receptor, either the human ER*α* (named as hER*α* assay) or Japanese medaka (*Oryzias latipes*) ER*α* (named as medER*α* assay), as well as co-activator TIF2 had been introduced. The estrogen activity is measured by the level of *β*-galactosidase activity.

Yeast cells, kindly provided by Dr. F. Shiraishi (National Institute for Environmental Studies, Japan), were pre-cultivated overnight at 30°C in synthetic defined (SD) medium free from tryptophan and leucine [26]. Yeast cell suspension was obtained from the overnight yeast cultures by diluting with SD medium lacking tryptophan and leucine to bring the $OD_{595}$ up to 1.65–1.8 $cm^{-1}$. The extract from each water sample was dissolved in 80 μl of DMSO and diluted in a geometric series. Each dilution (60 μL) was mixed with yeast cell suspension (120 μL) in a well of a 96-well polystyrene-made microplate (blackcolored type) (SUMILON, Sumitomo Bakelite, Tokyo, Japan) and incubated at 30°C for 4 h. Each well received 80 μL of a mixed solution containing 2 mg/L of Zymolyase-20 T solution (Seikagaku, Tokyo, Japan) for enzymatic digestion and light emission accelerator solution (Aurora Gal-XE kit; ICN Biomedicals, California, USA) for inducing chemiluminescence at the ratio of 5 : 3 (v/v) before another incubation at 37°C for 1 h. The intensity of chemiluminesence produced by the released *β*-galactosidase was measured with a luminometer (Luminescencer-JNR AB-2100; ATTO Bio-instrument,

Tokyo, Japan). Estrogenic activity was recorded as $EC_{10}$, which was defined as the concentration of the sample solution producing a chemiluminescent signal ten times that of the blank control. The inverse of the obtained $EC_{10}$ values of 17$\beta$-estradiol was set to 100.

The use of hER$\alpha$ and medER$\alpha$ assay in combination can be used to suggest causes of estrogenicity, as the medER$\alpha$ has higher affinity for xenobiotics than the hER$\alpha$ [27]. A larger estrogenic response in the medER$\alpha$ can suggest the presence of xenobiotic chemicals in samples, while a similar response in each assay suggests the activity is more likely due to estrogens.



### 2.7 *Laboratory study on filamentous green algae*

Laboratory experiments were designed to determine the effect of filamentous green algae (*Spirogyra* sp.) on the removal of estrogens. *Spirogyra* sp. collected from the field constructed wetland mesocosms was pre-cultivated in beakers containing the liquid medium recommended by OECD [28] in a temperature-controlled room at $22 \pm 1^{\circ}C$. The beakers were illuminated by cool white fluorescent tubes to give 100 μmol of photons $m^{-2} s^{-1}$ photosynthetically active radiation (PAR) on a 16: 8 h light : dark cycle.

Prior to experiments, *Spirogyra* sp. was disinfected by immersing them in NaClO (1%, v/v) for 3–5 min, rinsed with distilled water, and then weighed, where biomass was determined after removal of excess water through a 10-min period of air-drying and blotting with paper towels. In the jar tests, *Spirogyra* sp. was inoculated into 1000 mL culture medium spiked with the stock solution of estrone. The stock solution of estrone was prepared in methanol and stored at $-30^{\circ}C$ in the dark. The final concentration of methanol in the culture medium did not exceed 0.2%.

The experiment consisted of three treatments. Treatment A (Control): Culture medium spiked with estrone (no *Spirogyra* sp.); Treatment B: *Spirogyra* sp. was cultivated in culture medium spiked with estrone; Treatment C: *Spirogyra* sp. was cultivated in culture medium spiked with not only estrone, but also two broad-spectrum antibiotics: chloramphenicol (CAP) and streptomycin (STR). The final concentration of CAP and STR in culture medium in Treatment C was 10 and 2 mg/L, respectively. The biomass of *Spirogyra* sp. in each beaker of Treatment B and C was $1.90 \pm 0.27$ g dry wt./L. Each treatment was performed in triplicate.

The solution in each beaker, containing a Teflon-coated magnetic stirring bar ($\Phi 5\,mm \times 20\,mm$), was agitated gently with a magnetic stirrer at about 100 rpm, which achieved a satisfied mixture of solution and did not disturb the stationary growth state of *Spirogyra* sp. Water samples were collected on designed intervals to determine the concentrations of estrone in culture medium by the method described above.

### 2.8 *Statistical analyses*

The data between different treatment groups in each measurement were compared statistically by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test if the ANOVA result is significant at $P < 0.05$. The statistical analyses were performed with SPSS 12.0.

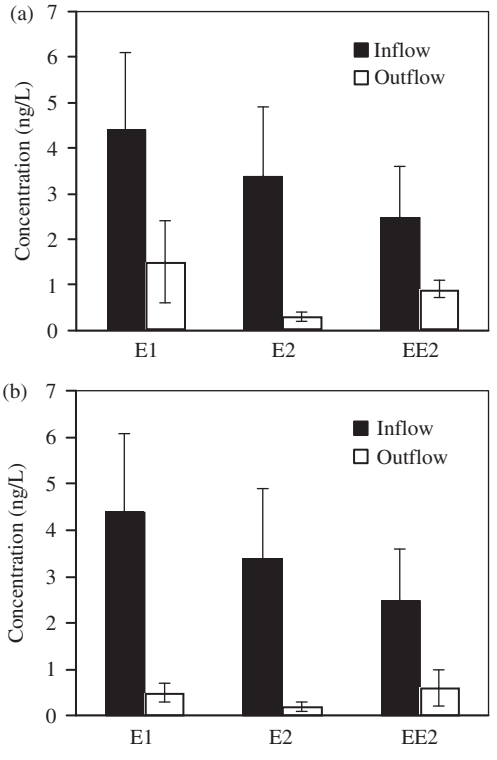



Figure 1. Concentrations of E1, E2 and EE2 in the inflow and outflow of the (a) SSF and (b) FWS CW mesocosms. Results are mean values (n = 10), and error bars represent the standard deviation.



## 3. Results and discussion

### 3.1 *Treatment performance*

Figure 1 shows the inflow and outflow concentrations of E2, E1 and EE2 for both SSF and FWS CWs. The concentrations of three estrogens measured in the Kamiyagari STW effluent were similar to the values reported at other STWs in Japan [29]. The concentrations of the target estrogens in outflow were found to be significantly lower than those in inflow for both SSF and FWS CWs ($P < 0.01$) (Figure 1).

Figure 2 shows the estrogenic activities of the inflow and outflow for both SSF and FWS CWs. Estrogenic activity was also comparable to those reported by previous studies for various STWs in Japan [30]. As shown in Figure 2, the estrogenic activity measured by the medER$\alpha$ assay was consistently nearly equal to that determined by the hER$\alpha$ assay for all water samples, indicating that it was estrogens but not xenobiotics that were responsible mostly for the estrogenic activity [27]. This result is in agreement with earlier reports that estrogens are major contributors to the estrogenic activity of the effluent [7,31]. In addition, similar to the attenuation of target estrogen compounds in CWs, the estrogenic activity was significantly lower at the outlet than at the inlet ($P < 0.01$) for both SSF and FWS CWs regardless of the assay method (i.e. either hER$\alpha$ or medER$\alpha$ assay).

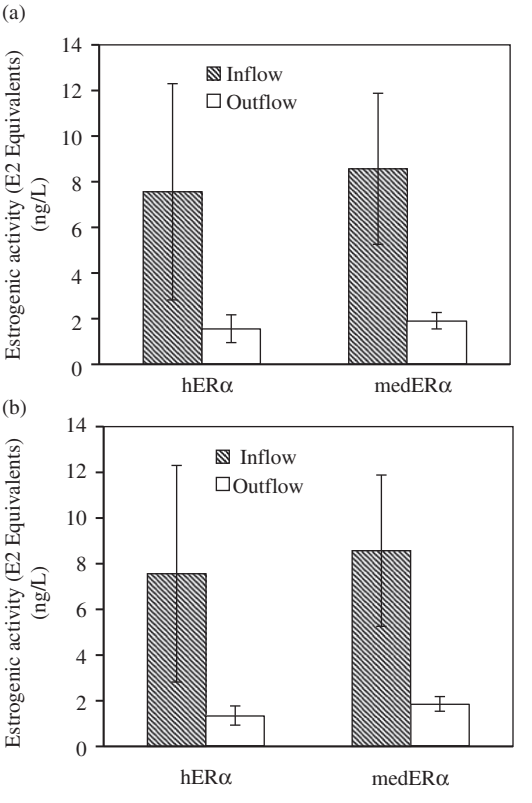



Figure 2. Estrogenic activity in water samples collected at the inlet and outlet of the (a) SSF and (b) FWS CW mesocosms. Estrogenic activity, expressed as the 17$\beta$-estradiol (E2) equivalent, was determined using the yeast two-hybrid assay (hER$\alpha$ assay: human ER$\alpha$ used as estrogen-receptor; medER$\alpha$ assay: Japanese medaka (*Oryzias latipes*) ER$\alpha$ used as estrogen-receptor). Results are mean values ($n = 10$), and error bars represent the standard deviation.



These results clearly show that, CWs, either subsurface flow or free water surface flow, were able to reduce simultaneously the estrogens and estrogenic activity in STW effluents. Also, our conclusions support the opinion of some other researchers who claimed that constructed wetlands were useful in the reduction of either estrogens or estrogenic activity in STW effluents [20,32]. Since CWs are increasingly being used as an alternative means of tertiary treatment stage following secondary treatment in STWs, our present results accompanied with other previous studies [20,32] suggest that the use of CWs may provide a relatively simple and inexpensive solution to deal with potential estrogenic contamination of STW effluents.

### 3.2 *Comparison of SSF and FWS CW*

The removal efficiency of target estrogen compounds and estrogenic activity in SSF and FWS CW is illustrated in Figure 3. On average, there was no significant difference ($P > 0.3$) between the removal rates achieved in the two different CW systems for each

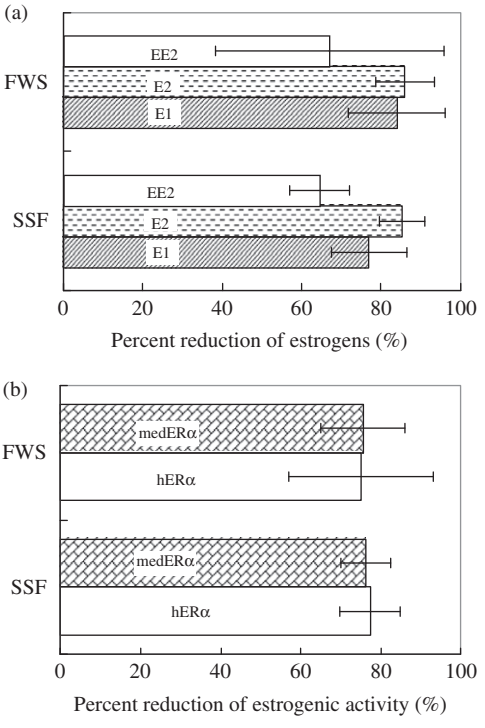



Figure 3. Comparison of the attenuation of (a) target estrogen compounds and (b) estrogenic activity in SSF and FWS CW mesocosms. Results are mean values ($n = 10$), and error bars represent the standard deviation.

of the estrogen compounds. Similarly, no significant difference was observed between the two different CW systems for the elimination of estrogenic activity, determined by either medER$\alpha$ assay or hER$\alpha$ assay. The present results clearly show that the performance of FWS CWs was comparable to SSF CWs with regard to the reducing estrogen compounds and estrogenic activity. On the contrary, it has been reported that the performance of SSF CWs in removing estrogens might be superior to that of FWS CWs [32]. However, it should be noted that this speculation has never been proven. Our results do not support the previous speculation.

Once estrogens have entered into CW systems, a series of processes, such as photolysis, biodegradation taking place in biofilms associated with substrate and plant surfaces, as well as sorption to bed sediments and plant biomass, can contribute to their elimination from the water phase. Given the relatively low polarity of these compounds, with octanol–water partition coefficients ($K_{ow}$) typically between $10^3$ and $10^5$ (Table 1), sorption to bed sediments and plant biomass is likely to be an important process. However, sorption is a physical removal process resulting only in sequestration of contaminants. The contaminants will ultimately break through the wetlands when the adsorption reaches saturation or equilibrium if transformation does not occur.

In fact, biodegradation of estrogens has been documented and recent research has focused on identifying estrogen-degrading strains. Estrogen-degrading bacteria have been found in various natural and engineered systems, such as activated sludge [33], marine sand [34], river water [35], soil [36] and compost [37], suggesting that such bacteria are

widespread in the environment. Therefore, some microorganisms capable of degrading estrogens are also very likely to be present in CWs.

As a consequence, the removal of estrogens in CWs is usually expected to be largely the result of a combination of sorption and biodegradation. However, if it had been the case in the present experiments, SSF mesocosms would have performed superior as compared to FWS mesocosms because SSF CW systems generally provide more surfaces for biofilm growth and more sites for physical adsorption than FWS CW systems. However, it was not the case in the present study.

There are two possible explanations for the similar performance of the two types of CW observed in our experiments. First, the comparatively more prevailing aerobic environment in FWS CW could offset the limited contact chance or time between water and microorganisms in comparison to SSF CW where the oxygen transport capacity of reeds is insufficient to ensure aerobic decomposition in the rhizosphere resulting in a dominant anoxic/anaerobic circumstance. It is well documented that the aerobic biodegradation of estrogens proceeds at a much faster rate than the anoxic and anaerobic biodegradation irrespective of either in aqueous phase or in solid phase. For example, the degradation rate of E1 in an activated sludge system decreased by a factor of between 3 and 5 in the transition from aerobic ($O_2$ available in solution) to anoxic (nitrate available but no molecular oxygen) as well as from anoxic to anaerobic [38]. Half-lives for the natural estrogens in aerobic sediment have been reported as <1 day but can be longer in anaerobic sediments with reports of up to 14 days for E1 and 21 days for E2 [35].

Second, besides the mechanisms responsible for the dissipation of estrogens in SSF CW systems, there are probably additional mechanisms in FWS CW systems contributing to the removal of estrogens. A distinct characteristic of FWS CW systems against SSF CW systems is that the water column in FWS systems is exposed to sunlight. Thus, the photo-degradation of estrogens becomes possible. Moreover, the exposure of water to sunlight often results in propagation of algae. Freshwater algae have been suggested to play an important role in the fate of organic compounds in the aquatic ecosystem although few studies have been devoted to examine the role of algae in the fate of estrogens. In fact, a noticeable growth of filamentous green algae (predominantly *Spirogyra* sp.) was observed in the water column in FWS CW mesocosms during the experiment.

In order to test the hypothesis that the growth of filamentous green algae together with the sunlight might be involved in the removal of estrogens, a field shading experiment as well as laboratory studies were further performed.



### 3.3 *Comparison of FWS CW with and without shading*

Comparison of FWS CW with and without shading was studied using two FWS CW mesocosms, one of which was inhabited by *Spirogyra* sp. in the open surface water column while another of which was cleaned up from *Spirogyra* sp. by hand and subsequently covered by strips of black plastic sheet avoiding the re-colonisation of filamentous green algae. Figure 4 presents the average removal efficiencies of target estrogen compounds and estrogenic activity in both FWS CW mesocosms with and without shading.

As can be seen in Figure 4, the removal efficiencies of target estrogen compounds in FWS CW mesocosms with shading were significantly lower than that in FWS CW mesocosms without shading ($P < 0.05$). Similar results were obtained for estrogenic activity regardless of either hER$\alpha$ or medER$\alpha$ assay. These results clearly demonstrated that

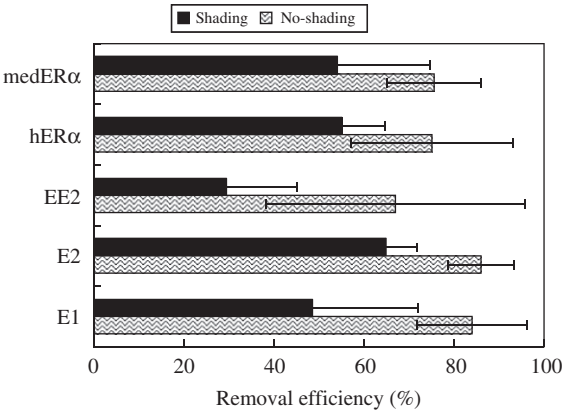



Figure 4. The removal of target estrogen compounds and estrogenic activity by FWS CW mesocosms with and without shading. Results are mean values ($n = 8$), and error bars represent the standard deviation.



getting rid of *Spirogyra* sp. accompanied with shading led to a notable decrease in the removal efficacy of FWS CW mesocosms. If we assume that, under the condition of no-shading and presence of filamentous green algae, the performance of the two experimental FWS CW mesocosms was the same, the contribution of filamentous green algae could be estimated based on a rough mass balance analysis. The calculation result shows that filamentous green algae accounts for approximately 42.2%, 24.4%, 55.2% and 27.0% of the total removed E1, E2, EE2 and estrogenic activity, respectively, in the FWS CW mesocosm without shading.

Furthermore, considering the performance of SSF CW was comparable to the performance of FWS CW without light-shading (i.e. with the presence of filamentous algae and algae-attached bacteria) as indicated in Section 3.2 above, it can be inferred that the efficiency of FWS CW in the case of without filamentous algae and algae-attached bacteria should be inferior to that of SSF CW with regard to the removal of estrogens and estrogenic activity. Therefore, it can be concluded that the growth of filamentous green algae together with the sunlight were responsible partially for the attenuation of estrogens and estrogenic activity in the FWS CW mesocosms inhabited by filamentous green algae without shading. Nonetheless, considering the experiment was still not able to distinguish the photolysis and algae-mediated dissipation, the role of *Spirogyra* sp. was evaluated specifically in the following section.

### 3.4 *Laboratory study on filamentous green algae*

In the laboratory study, E1 was selected as the target compound due to its widespread presence in wastewater effluents as a result of the relatively low removal in STW, and because it is a major and relatively persistent biotransformation product of E2 [8,29,38]. The removal of E1 was studied under batch conditions with hydroponic cultures of *Spirogyra* sp. As can be seen in Figure 5, there was a sharp decrease of E1 concentration at the beginning for both Treatments A (control) and B, which could be accounted for by the rapid adsorption of E1 to the glass beaker. However, a notable difference was observed

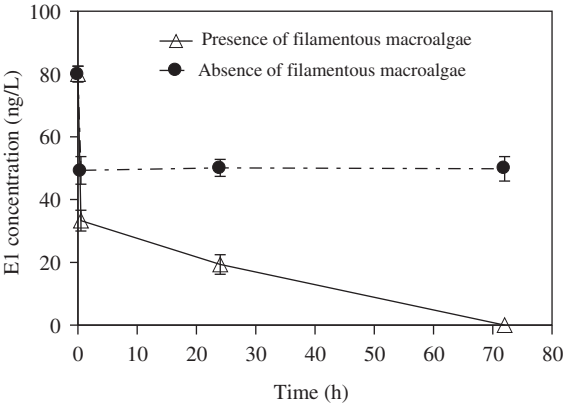



Figure 5. The change in E1 concentration in the culture solutions with the presence or the absence of filamentous green algae (*Spirogyra* sp.). Data points show averages of three replicates of each treatment. Error bars indicate standard deviation of the triplicates.

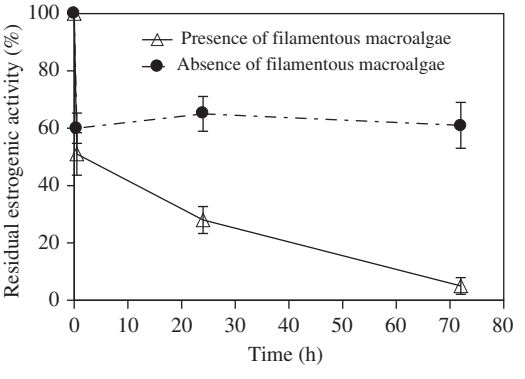



Figure 6. The percentage residual estrogenic activity determined by yeast two-hybrid assay (hER$\alpha$ assay) in the solutions as a function of time in culture solutions with the presence or the absence of filamentous green algae (*Spirogyra* sp.). Data points show averages of three replicates of each treatment. Error bars indicate standard deviation of the triplicates.



during the following period. The concentration of E1 persisted in Treatment A, whereas gradually declined to below the detection limit in Treatment B until the end of the experiment. This result indicated that the presence of algae was capable of enhancing the removal of E1. In addition, this result also suggested that the direct photolysis of E1 seemed unlikely in the growth medium without algae due to the persistence of E1 in the control treatment (Treatment A), at least during the time span we employed.

Figure 6 shows the residual estrogenic activity in the culture solutions as a function of time determined by YTA using human ER$\alpha$ as estrogen-receptor (hER$\alpha$ assay). There was an obvious reduction in the estrogenic activity with the lapse of time in the hydroponic culture of *Spirogyra* sp. In contrast, following a rapid drop due likely to adsorption effect of the glassware, the estrogenic activity in the culture solutions with no filamentous algae remained constant over the experiment period. Similar results were obtained with medER$\alpha$

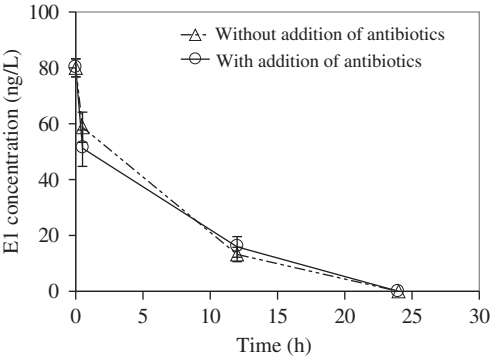



Figure 7. Concentration profile of E1 in the solutions of *Spirogyra* sp. culture systems with and without the addition of two broad-spectrum antibiotics, chloramphenicol (CAP) and streptomycin (STR). Data points show averages of three replicates of each treatment. Error bars indicate standard deviation of the triplicates.



assay (data not shown). Therefore, it can be deduced that the observed degradation of E1 by *Spirogyra* sp. corresponded directly to a reduction in estrogenic activity.

Nonetheless, the possibility still existed that algae-associated bacteria contributed to some extent to the dissipation of E1 in treatment B considering that the experiments were not performed under aseptic conditions. On the suspicion the microbial degradation by algae-attached bacteria might account for the reduction of E1, microbial inhibition experiments were additionally carried out by concurrently using two broad-spectrum antibiotics (i.e. Treatment C). The results are presented in Figure 7. It can be seen from Figure 7 that the addition of two broad-spectrum antibiotics had little effect on the decline of E1 in the algae culture solutions. Therefore, it is unlikely that the decreases in the concentrations of E1 in *Spirogyra* sp. culture solutions were attributable to the degradation by bacteria.

Collectively, it was very likely that *Spirogyra* sp. itself rather than algae-associated bacteria was responsible for the removal of E1. To the best of our knowledge, the present result provides the first proof that *Spirogyra* sp. has the ability of reducing estrogens.

It has been reported that procaryotic and eucaryotic photoautotrophic algae were capable of biotransforming and biodegrading organic micropollutants commonly found in natural and waste waters. For example, a freshwater green alga, *Selenastrum capricornutum*, can metabolise benzo[a]pyrene (BaP) through a dioxygenase pathway with subsequent conjugation and excretion [39]. However, it still remains unclear what mechanism is exactly related to the removal of estrogens by *Spirogyra* sp., such as assimilation, uptake, irreversible sorption and photo-degradation induced by algal derived dissolved organic carbon, and so on. The study on mechanisms will be helpful not only to elucidate the fate of estrogens in engineered as well as natural aquatic systems, but also to resolve the concern about whether estrogens can be bio-accumulated by filamentous green algae resulting possibly in a significant body burdens at subsequent trophic levels. This is a topic worthy of future investigation. In view that filamentous green algae are commonly observed in a variety of water environments, more attention should be paid to this algae species when conducting research on the fate of other emerging organic pollutants besides estrogens in aquatic environments.

## 4. Conclusion

Under field conditions, both the SSF and FWS CW mesocosms were able to efficiently reduce the estrogens and estrogenic activity in an STW effluent. In addition, there was no significant difference between the SSF and FWS CW mesocosms in terms of the removal ability of estrogens and estrogenic activity.

Abundance of filamentous green algae, predominantly *Spirogyra* sp., was observed growing in overlying water column in FWS CW mesocosms. Clearing away *Spirogyra* sp. followed by shading resulted in a remarkable decrease in the removal of estrogens and estrogenic activity for the FWS CW mesocosms. The results suggested that *Spirogyra* sp. and the sunlight contributed greatly to the removal ability of FWS CW mesocosms for estrogens and estrogenic activity during the growing season of filamentous green algae, which can help to explain the comparable performance of the SSF and FWS CW mesocosms.

Furthermore, the results of filamentous green algae culture experiments confirmed that it was *Spirogyra* sp. but not algae-associated bacteria that were directly related to the removal of estrogens. However, the mechanisms involved in the removal of estrogens by *Spirogyra* sp. remain unknown, which opens a new window for future research.



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