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Biotransformation of progesterone and norgestrel by two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*): Transformation kinetics and products identification

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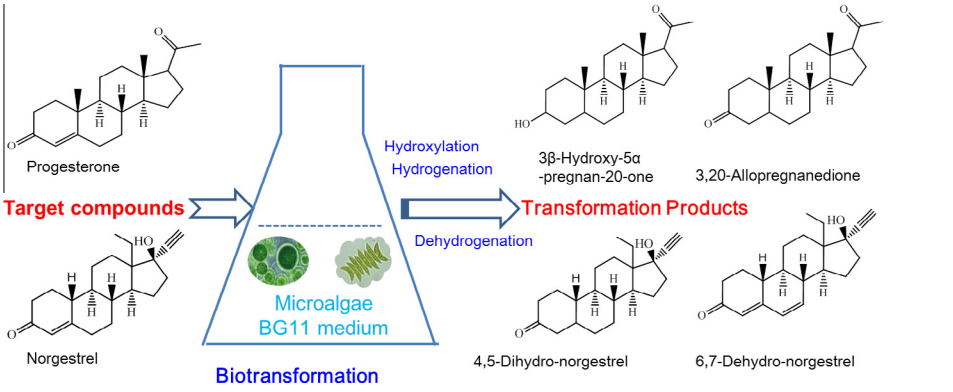
HIGHLIGHTS

- Degradation of progesterone and norgestrel by two algae was investigated.
- Progesterone was degraded more rapidly than norgestrel within 5 d.
- The algae *S. obliquus* and *C. pyrenoidosa* performed differently in the degradation.
- Some transformation products were identified for progesterone and norgestrel.
- Hydroxylation, reduction and oxidation were involved in the degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Natural and synthetic steroid hormones such as progesterone and norgestrel in the aquatic environment may cause adverse effects on aquatic organisms. This study investigated the biotransformation of progesterone and norgestrel in aqueous solutions by two freshwater microalgae *Scenedesmus obliquus* and *Chlorella pyrenoidosa* and elucidated their transformation mechanisms. More than 95% of progesterone was transformed by the two microalgae within 5 d. For norgestrel, almost complete transformation by *S. obliquus* was observed after 5 d, but nearly 40% was remained when incubated with *C. pyrenoidosa*. The results also showed that these two compounds were not accumulated in the algal cells. Biotransformation was found to be the main mechanism for their loss in the aqueous solutions, and it followed the first-order kinetic model. For progesterone, three main transformation products, i.e. 3β-hydroxy-5α-pregnan-20-one, 3,20-allopregnanedione and 1,4-pregnadiene-3,20-dione, and six minor androgens were identified. For norgestrel, only two transformation products, 4,5-dihydronorgestrel and 6,7-dehydronorgestrel, were identified for the first time. Hydroxylation, reduction and oxidation are proposed to be the main transformation pathways. Among the two microalgae species, *S. obliquus* was found more efficient in the transformation of the two target compounds than *C. pyrenoidosa*. The results clearly demonstrated the capability of the two microalgae to transform the two progestogens. The biotransformation and products could have significant environmental implications in the fate and effects of the two steroids.

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1. Introduction

Steroid hormones in the aquatic environment have become a big concern due to their high potency and potential adverse effects on aquatic organisms (Lintelmann et al., 2003; Runnalls et al., 2010). Feminization of male fish in some rivers and lakes has been reported due to the presence of natural and synthetic estrogens (Jobling et al., 1998; Kidd et al., 2007; Xie et al., 2010). So far, most attention has been directed to studying the environmental fate and biological effects of estrogens and less to the other steroids such as progestogens (Ying et al., 2002; Sumpter, 2005; Chang et al., 2011).

Since little has been known about the fate of progestogens in the aquatic environment, this study will focus on two progestogens: one is progesterone, a natural steroid hormone, which is a key compound in regulating female reproductive function; the other is norgestrel, a synthetic compound, which is often used as active ingredient in oral contraceptives. Progesterone and norgestrel have been detected in sewage effluents and receiving waters at several to hundreds ng L^{-1} levels (Kolpin et al., 2002; Chang et al., 2011; Liu et al., 2011, 2012). Recent laboratory studies showed that progesterone and norgestrel could affect fish reproduction and hormone receptor gene expressions at ng L^{-1} levels (Zeilinger et al., 2009; DeQuattro et al., 2012; Zucchi et al., 2012). Some investigations from pharmaceutical industry indicate that steroids including progesterone and norgestrel could be transformed by algae, bacteria and fungi (Hu et al., 1996; Pollio et al., 1996; Huszcza et al., 2005; Eshrat and Aroona, 2011). However, in-depth studies are needed to understand the role of various microbes like algae in the fate of progesterone and norgestrel in the aquatic environment, including their transformation mechanisms and transformation products.

Algae play an important role in the environmental fate of contaminants since they are widespread in the aquatic environment and can interact with organic and inorganic substances (Field et al., 1998; Munoz and Guieysse, 2006; Gao and Tam, 2011; Zhou et al., 2011). It is known that like bacteria and fungi, algae are able to remove organic contaminants via adsorption, absorption and degradation processes (Hirooka et al., 2005; Nakajima et al., 2007; Li et al., 2009; Gao and Tam, 2011). The objectives of this study are to assess the biotransformation potential of two progestogens (progesterone and norgestrel) by two widely distributed freshwater green microalgae *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, and to investigate their algal transformation mechanisms and transformation products. The two algae species used in this study have been used in our previous investigations for other contaminants including metals and alkyl phenols (Zhou et al., 2011, 2012). The results from this study are expected to facilitate better understanding the potential application of freshwater algae in wastewater treatment and their interaction with the progestogens in the aquatic environment.

2. Materials and methods

2.1. Chemicals and materials

Progesterone (CAS: 57-83-0; purity: 99%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, German), while norgestrel (CAS: 6533-00-2; purity: 99%) was obtained from USP (Rockville, MD). The stock solutions of these two target compounds were prepared in methanol at the concentration of 6369.4 μM. Twenty-eight steroid standards were also obtained from various suppliers for analysis and confirmation of various transformation products (Liu et al., 2011). In addition, 3β-hydroxy-5α-pregnan-20-one and 3,20-allopregnanedione were purchased from Sigma–Aldrich (St. Louis, MO), and used as the qualitative standards for confirmation. Mineral salts such as $NaNO_3$, Na_2CO_3 and $K_2HPO_4{\cdot}3H_2O$ (analytical grade) were obtained from Tianjin (China) and used for preparation of algal growth medium. Formic acid (HPLC grade, purity ⩾98%) was supplied by Tedia Company (Tedia, USA). All the organic solvents used in extraction and analysis (acetonitrile, methanol, methylene dichloride, hexane and ethyl acetate) were HPLC grade and purchased from Merck Corporation (Shanghai, China) or CNW Technologies (Dusseldorf, Germany). Ultrapure water was produced by a Milli-Q apparatus from Millipore (Watford, UK). Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Supelco (USA) to be used as derivatization reagent. Oasis HLB cartridges (500 mg) were obtained from Waters Corporation (Milford, MA, USA). All glassware was hand-washed with tap water, rinsed with methanol and HPLC grade water, and backed at 450 °C for more than 4 h before use when necessary.

2.2. Microalgae and culture medium

C. pyrenoidosa (FACHB-9) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), while *S. obliquus* was isolated from a hypereutrophic environment (the East Lake, Wuhan, China). BG11 medium, described by Stanier et al. (1971), was used as the growth medium. The growth medium contained the following chemicals: $NaNO_3$, 1.5 g L^{-1}; $K_2HPO_4{\cdot}3H_2O$, 0.04 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 0.075 g L^{-1}; $CaCl_2{\cdot}2H_2O$, 0.036 g L^{-1}; Na_2CO_3, 0.02 g L^{-1}; citric acid, 0.006 g L^{-1}; ferric ammonium citrate, 0.006 g L^{-1}; EDTA, 0.001 g L^{-1}; and A_5+Co solution (1 ml L^{-1}) that consists of H_3BO_3, 2.86 g L^{-1}, $MnCl_2{\cdot}4H_2O$, 1.81 g L^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.222 g L^{-1}; $CuSO_4{\cdot}5H_2O$, 0.079 g L^{-1}; $Na_2MoO_4{\cdot}2H_2O$, 0.390 g L^{-1}; and $Co(NO_3)_2{\cdot}6H_2O$, 0.0494 g L^{-1}. The two selected algae species were cultivated in 150 mL BG11 medium in flasks, which were placed in a shaking incubator with a speed of 150 rpm at 25 °C. Illumination was provided by white fluorescent light at 3000 lux with the light/dark cycle of 12 h:12 h. Algal cells used for the transformation experiment were collected by centrifuging the culturing solution at the exponential growth phase, then pellets were harvested, and washed three times with Milli-Q water and re-suspended in 100 mL Milli-Q water.

2.3. Biotransformation experiment

The biotransformation experiment of progesterone and norgestrel by *C. pyrenoidosa* and *S. obliquus* was conducted individually under strict axenic conditions. The experimental design is shown in Fig. S1 (Supporting Information). The target compound (progesterone or norgestrel) in the stock solution was added into the BG11 medium to achieve an initial concentration of 1.6 μM. Algal cells were inoculated at an initial algae density of approximately 10^5 or 10^6 cells mL^{-1}. Culturing was performed in 250 mL Erlenmeyer flasks containing 150 mL culture volume at 150 rpm and 25 ± 1 °C. Light was provided by continuous cool white fluorescent lamps at 3000 lux with a dark/light cycle of 12 h:12 h. The experiment was conducted for 5 d, and both treatments were carried out in triplicate for the degradation kinetics. The treatments for both progesterone and norgestrel had corresponding controls without the target chemicals (only live algae) to assess chemical effect on algal growth, and without algae and with dead algae to measure the abiotic losses (and/or adsorption) of the target compounds from the culture medium. Dead algae were achieved by heat treatment (85 °C, 20 min) (Majidi et al., 1990). The dead algae were added to the culture at an initial density of 10^6 cells mL^{-1}. The concentrations of the two target compounds in the aqueous culture solutions and algal cells were monitored at different time intervals (0, 5, 10, 24, 48, 72, 96 and 120 h) within the 5 d incubation. Separate batches of culture flasks with the same treatments were also set

up for the algal absorption measurement and transformation products identification.

2.4. Determination of algae density

The cell density in each treatment was measured daily with optical density (OD) at 650 nm or 680 nm for the growth of *C. pyrenoidosa* and *S. obliquus* respectively, using a BMG microplate reader (BMG Lab technologies, Offenburg, Germany). The relationship between algal density and OD_{650} or OD_{680} is given in Eqs. (1) and (2) (Zhou et al., 2011).

For *C. pyrenoidosa*:

$$\text{Cell density}\ \ (10^5\ \text{cells mL}^{-1}) = 334.74 \times OD_{680} - 6.6694 \quad (R = 0.997) \tag{1}$$

For *S. obliquus*:

$$\text{Cell density}\ \ (10^5\ \text{cells mL}^{-1}) = 78.953 \times OD_{650} - 1.9331 \quad (R = 0.995) \tag{2}$$

$$\text{Dry weight}\ \ (\text{mg mL}^{-1}) = 0.7244 \times OD_{650} - 0.0176 \quad (R = 0.999) \tag{3}$$

2.5. Analysis of progesterone and norgestrel

The concentrations of the target compounds (progesterone and norgestrel) in the culture solutions for the transformation kinetics were analyzed at the different time intervals by high-performance liquid chromatography (HPLC). Exactly 1.5 mL of the culture solutions was sampled each time and centrifuged at 9168*g* for 5 min to remove the algal cells, and then 1 mL of the supernatant was extracted three times with 0.3 mL methylene dichloride. The extracts were pooled together and re-constituted in the initial mobile phase solutions for determination of the progesterone or norgestrel concentrations.

The progesterone and norgestrel adsorbed/absorbed in algal cells were also determined daily from a different batch of flasks with the same treatments to those for the transformation kinetics. Algae cells were harvested from 150 mL algae culture medium by centrifugation at 9168*g* for 5 min, then re-suspended in 1.5 mL of 10% methanol aqueous solution and shaken for approximately 30 s to obtain the progesterone and norgestrel adsorbed on the cell walls. The sample solution was then centrifuged for a further 5 min at 9168*g*, and the target compound contained in the supernatant was used for the determination of the progesterone or norgestrel content adsorbed on the algal cells (Correa-Reyes et al., 2007; Zhou et al., 2012). The algal cells after 10% methanol wash were frozen (−20 °C) over night until the cell walls were broken. The processed algae were then extracted three times with dichloromethane–methanol (1:2 v/v) to obtain the progesterone and norgestrel absorbed in algal cells (Correa-Reyes et al., 2007; Zhou et al., 2012).

The HPLC instrument used in the measurement was an Agilent HPLC system (1200 series) equipped with a diode-array detector (DAD) (Agilent Technology, Santa Clara, CA). A Zorbax Eclipse reversed-phase XDB-C18 (4.6 × 150 mm, 5 μm) was used for the separation of the target compounds under the oven temperature of 30 °C. The mobile phase was 80% acetonitrile and 20% water for progesterone, and 60% acetonitrile and 40% water for norgestrel, with a flow rate of 1 mL min^{-1}. The injection volume was 20 μL and the UV wavelength used for detection was 240 nm for both target compounds. The method limits of quantification (MLOQs) for progesterone and norgestrel were 44.6 μg L^{-1} and 43.5 μg L^{-1}; and their recoveries from aqueous solutions were 84.5 ± 2.3% and 98 ± 7.1%, respectively.

Based on the measured concentrations, the transformation efficiency (R) and the bioconcentration factor (BCF) were calculated according to the following Eqs. (4) and (5) (Zhou et al., 2012).

$$R = 100 \times (C_i - C_f)/C_i \tag{4}$$

where R is the dissolved progesterone or norgestrel removal (%); C_i and C_f are the initial and final concentrations (mg L^{-1}) of progesterone or norgestrel in the solution, respectively.

$$\text{BCF} = C_c/(C_i/1020) \tag{5}$$

where C_c is the concentration (mg g^{-1} dry weight) of progesterone or norgestrel accumulated in the algal cells, C_i is the initial concentrations (mg L^{-1}) of progesterone or norgestrel in the solution, divided by 1020 g L^{-1} as the density of the solution.

2.6. Analysis of transformation products

Solid phase extraction (SPE) was used to extract potential transformation products from both treatments for the two target compounds (progesterone and norgestrel), with a method modified from our previous work (Liu et al., 2011). The culture solutions (150 mL each) sampled every day during the 5 d incubation were centrifuged at 9168*g* for 5 min, and then the supernatants were passed through the Oasis HLB cartridges which had been pre-conditioned with 10 mL methanol and 10 mL Milli-Q water. The potential transformation products from progesterone or norgestrel were eluted sequentially with 10 mL hexane, followed by 10 mL ethyl acetate and 10 mL methanol. The extracts were dried under a gentle nitrogen stream and re-dissolved in 1 mL of methanol and stored in a fridge for later analysis. The collected algal cells were also extracted with dichloromethane–methanol (1:2 v/v) to obtain transformation products that may be absorbed in the algal cells. The extracts were analyzed by both gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS).

The transformation products of the two progestagens by algae were identified by the following three main methods: GC–MS scan analysis to obtain mass spectra, LC–MS/MS scan analysis (Scan mode) to obtain the molecular weights of the products and fragmentation patterns, and LC–MS/MS quantitative analysis with multiple reaction monitoring mode (MRM mode) to confirm and quantify the potential degradation products by using available authentic standards.

The GC–MS instrument used in the analysis of transformation products was an Agilent 6890N gas chromatograph (Agilent, USA) connected to an Agilent 5975B MSD mass spectrometer. Separation of the compounds was achieved by using a DB-5ms capillary column (30 m × 0.25 mm, 0.25 μm film thickness) (Agilent J&W, USA). Helium was used as the carrier gas and maintained at a constant flow rate of 1.0 mL min^{-1}. A sample volume of 2 μL was injected in the splitless mode at 280 °C. The column temperature was programmed from 80 °C to 310 °C at 5 °C min^{-1}, and then maintained at 310 °C for 5 min. The mass spectrometer was operated under electron ionization (EI) mode with a mass scan range of 40–1000 amu. The ion source temperature was kept at 250 °C and the quadrupole temperature 150 °C during the analysis. Compound identification was carried out based on mass spectra interpretation, chemical standard match and/or NIST mass spectra library searching program (database version 05 from Institute of Standards and Technology).

The extracts (100 μL each) were also derivatized using 20 μL acetic ether, 50 μL pyridine and 30 μL BSTFA. Conversion into their trimethylsilyl (TMS) derivatives was achieved by heating the reaction solutions at 70 °C for 60 min, and the reaction solutions were then dried by a gentle nitrogen stream and re-constituted in ethyl

acetate. Silylated compounds in the extracts were again analyzed by GC–MS for any polar transformation products.

The LC–MS/MS instrument used in the analysis of potential transformation products was an Agilent 1200 LC-Agilent 6460 QQQ with an electrospray ionization (ESI) source. The chromatographic separation was performed on an Agilent Zorbax SB-C18 (100 mm × 3 mm, 1.8 μm) column with its corresponding pre-column filter (2.1 mm, 0.2 μm). Two RRLC–MS/MS analysis methods were applied in the identification: one used the Scan method to confirm the product ions and parent ions (pseudomolecular ions), and the other used our previous quantitative method (multiple reaction monitoring method (MRM method)) to confirm and quantify potential transformation products with available authentic standards (Liu et al., 2011). The mobile phase used in the analysis was: water containing formic acid (0.01%, v/v) and methanol at a flow rate of 0.35 mL min^{-1}. The gradient program used in the Scan method was as follows: 60% methanol for 50 min, and post time for 5 min, while that for the MRM method was given as follows: from 60% to 80% (methanol) in 15 min, then from 80% to 60% (methanol) in 0.5 min, and post time for 5 min. Both methods were performed in positive ionization mode.

3. Results

3.1. Biotransformation of progesterone and norgestrel by microalgae

Growth of the two freshwater microalgae during the transformation of progesterone and norgestrel is shown in Fig. S2 (Supporting Information). The two algae *S. obliquus* and *C. pyrenoidosa* maintained a constant growth in the presence of progesterone or norgestrel. No significant effects were observed between the treatments with an initial chemical concentration of 1.6 μM and the controls without the target compounds ($p < 0.05$).

Both of the target compounds (progesterone and norgestrel) were found to be stable under the sterile conditions. Any degradation due to hydrolysis or volatilization was negligible based on the data for the sterile controls. These two compounds were degraded significantly by the two live algae within120 h (5 d) of incubation (Figs. 1 and 2). The biotransformation of progesterone and norgestrel by the live algae followed the first-order reaction model. The kinetic parameters including the kinetic rate constant (k) and the half-life ($t_{1/2}$) are given in Table 1.

But only slight losses of progesterone and norgestrel by the dead algae (<20%) were found within 120 h, and the losses occurred mainly within 24 h (Figs. 1 and 2). Thus, adsorption is the only contribution factor for the removal under the dead algae treatment. This suggests that the removal of progesterone and norgestrel in the aqueous solutions by live *S. obliquus* and *C. pyrenoidosa* was caused mainly by biotransformation rather than by simple accumulation by algal cells. It should be noted that more adsorption may occur with increasing biomass during the incubation of live algae.

In fact, the two target compounds in live algal cells were also determined in the experiment. Progesterone, whether adsorbed or absorbed, was not found in the live algal cells, while norgestel was only detected to be absorbed in *S. obliquus* cells at the 3rd, 4th and 5th day with its trace concentrations of 23 ± 0.5, 24 ± 0.5 and 17 ± 0.6 mg kg^{-1}. The corresponding BCF for the three sampling days were calculated to be 48 ± 1.0, 50 ± 1.0 and 36 ± 1.2, respectively. Therefore, biotransformation was the main process responsible for the losses of these two progestogens in the aqueous solutions.

For progesterone, more than 95% was degraded from the aqueous medium by the two live microalgae species at the end of the experiment (120 h) (Fig. 1). However, *S. obliquus* and *C. pyrenoidosa* performed differently in the degradation process. Rapid transformation of progesterone by *S. obliquus* was observed within the first 48 h, followed by a slow transformation. In comparison with *S. obliquus* ($t_{1/2}$: 16 h), slower transformation by *C. pyrenoidosa* was found for progesterone with its half-life of 39 h (Table 1).

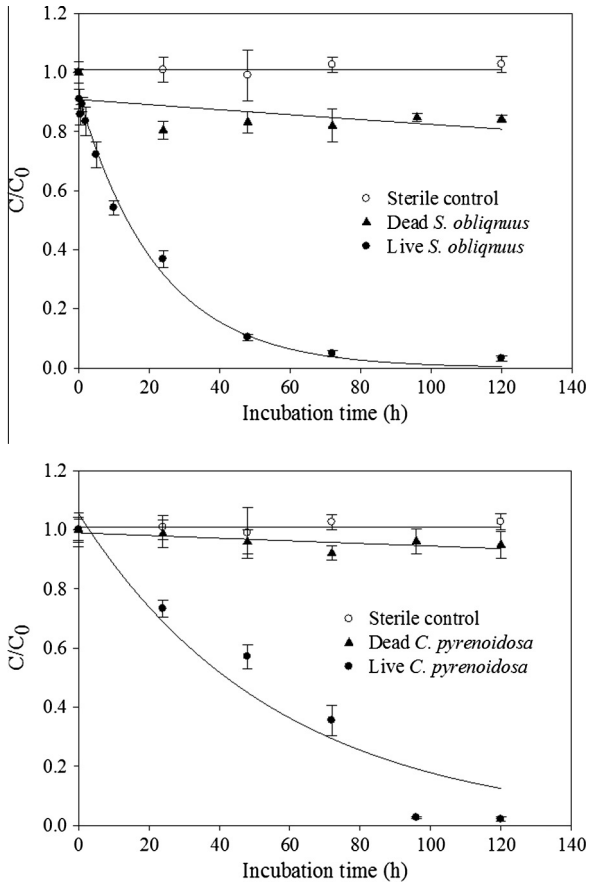

Fig. 1. Transformation of progesterone (initial concentration of 1.6 μM) by *S. obliquus* and *C. pyrenoidosa*. Error bars indicate standard deviations of the residual concentrations ($n = 3$).

Norgestrel biotransformation was found much slower than that of progesterone by either *S. obliquus* or *C. pyrenoidosa* (Fig. 2). The half-lives for norgestrel were 40 h with *S. obliquus* and 88 h with *C. pyrenoidosa* (Table 1). It is noted that *S. obliquus* was more effective than *C. pyrenoidosa* in the transformation of both progesterone and norgestrel although both algae could transform the two compounds.

3.2. Identification of the transformation products

Three main and six minor transformation products were tentatively identified for progesterone by using GC–MS and LC–MS/MS (Table 2). The three main transformation products, i.e. 3β-hydroxy-5α-pregnan-20-one (**A**), 3,20-allopregnanedione (**B**) and 1,4-pregnadiene-3,20-dione (**C**) were first identified by GC–MS with NIST library match (Fig. S3), and products **A** and **B** were also confirmed by their pure chemical standards (Figs. S4 and S5). Product **A** (3β-hydroxy-5α-pregnan-20-one) had a molecular ion at m/z 318.2 and characteristic fragment ions at m/z 215.1, 235.2, 257.1, 285.2 and 298.2. Product **B** had a molecular ion at m/z 316.2, and other characteristic fragment ions at m/z of 231.1, 258.2 and 298.2. Product **C** had a molecular ion at m/z of 312.2 and a characteristic fragment ion at m/z of 122. The three main transformation products (**A**, **B**, and **C**) of progesterone were detected in the

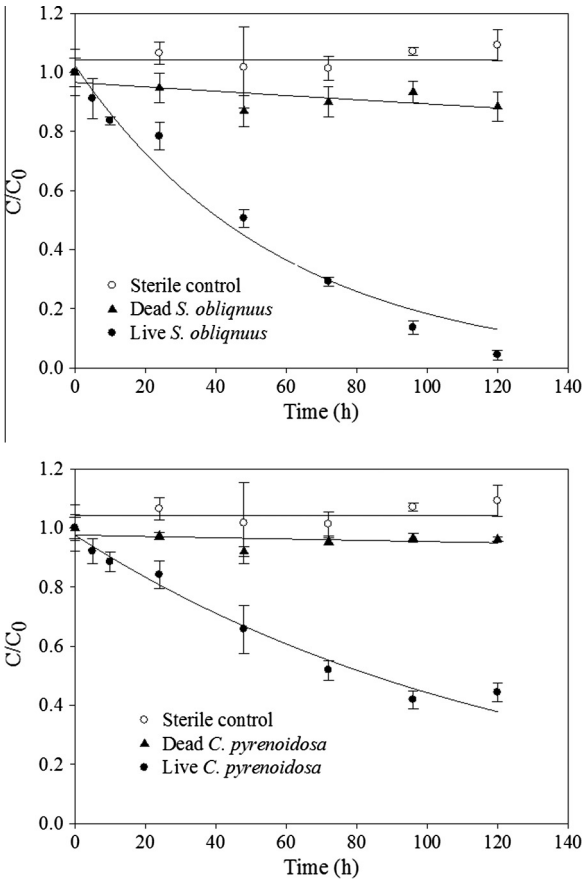

Fig. 2. Transformation of norgestrel (initial concentration of 1.6 μM) by *S. obliquus* and *C. pyrenoidosa*. Error bars indicate standard deviations of the residual concentrations ($n = 3$).

incubation solution with *C. pyrenoidosa*, while only one main product (**C**) was detected in both algae culture media (Fig. S6). The three main products (**A**, **B** and **C**) were also confirmed for the presence of their pseudomolecular ions (M+H: 319, 317 and 313) by the LC–MS/MS with Scan mode. Based on the peak areas, the yields for the three main products (**A**, **B** and **C**) were estimated to 21.8%, 3.5% and 18.7% after 5 d incubation of *C. pyrenoidosa* (Table S1).

The six minor transformation products for progesterone were identified by LC–MS/MS with the authentic standards based on our previous quantitative method for various steroids (Liu et al., 2011). The six products (**D** to **I**) at the ng L^{-1} to μg L^{-1} levels (Table S2) were androgen steroids, including ADD (androsta-1,4-diene-3,17-dione), 17β-boldenone, AED (4-androstene-3,17-dione), testosterone, 5α-DHT (5α-dihydrotestosterone), and epi-androsterone (Table 2). They could be found in both incubation media of *S. obliquus* and *C. pyrenoidosa* with progesterone.

For norgestrel, only two transformation products 4,5-dihydronorgestrel (**J**) and 6,7-dehydronorgestrel (**K**) were tentatively identified based on the mass spectra from GC–MS analysis of the derivatized extracts, and further confirmed by the NIST library match (Fig. S7). Silylated product **J** had a molecular ion (M+TMS) at m/z of 386.3, while silylated product **K** had a molecular ion (M+TMS) at m/z 382.3, which correspond to their molecular weights of 314 and 310. The two products (**J** and **K**) were only found for norgestrel incubated with *C. pyrenoidosa* (Table 2), with their yields estimated to be 47% and 10% after 5 d incubation, respectively. All identified transformation products for progesterone and norgestrel were detected in the incubation solutions, not inside the algal cells. Based on the detected transformation products, it is found that hydrogenation and dehydrogenation were the two main mechanisms for the algal transformation of progesterone and norgestrel. The biotransformation pathways for the two compounds were tentatively proposed and are shown in Fig. 3.

4. Discussion

The results from the present study showed the capability of the two freshwater microalgae, *S. obliquus* and *C. pyrenoidosa*, to degrade the two progestogens progesterone and norgestrel (Figs. 1 and 2). The two algae species showed different metabolisms, with *S. obliquus* having better biotransformation capability than *C. pyrenoidosa* for the two progestogens. A previous pharmaceutical study of ten different microalgae strains by Pollio et al. (1994) found that the Chlorococcales and Cyanidium groups were the most promising strains to bioconvert progesterone, while the *Volvocaceae* and *Euglenophyceae* groups did not induce any transformation. The half-lives for the two compounds in Table 1 clearly suggest easier biotransformation for the natural progestogen progesterone than for the synthetic progestogen norgestrel by both algae species. This is most likely due to the presence of 17α-ethynyl group at the molecular structure of norgestrel, which makes it more resistant to microbial attack (Hu et al., 1998).

Algal transformation of progesterone produced three main transformation products, i.e. 3β-hydroxy-5α-pregnan-20-one (**A**), 3,20-allopregnanedione (**B**) and 1,4-pregnadiene-3,20-dione (**C**) as well as six minor androgen compounds (**D**–**I**) (Table 2). The reactions involved in the algal transformation of progesterone include the reduction (hydrogenation), hydroxylation, oxidation (dehydrogenation) and side-chain breakdown (Fig. 3). Similar transformation pathways for progesterone by some strains of *Chlorella* spp. have been previously observed (Pollio et al., 1996). Different metabolisms among these investigated strains were also found in their study. Some products such as products **A** and **B** detected in the present study due to the hydroxylation and reduction of progesterone were also found in their study. Hydroxylation of progesterone has been reported as the main transformation pathway by a terrestrial-isolated cyanobacterium *Microchaete tenera* with the formation of 6-hydroxy and 20-hydroxyl derivatives (Safiarian

Table 1
Kinetic parameters for the algal transformation of progesterone and norgestrel.

Compound[a]	CAS	Molecular weight	Algae species	k (h)[b]	r^2[c]	$t_{1/2}$ (h)[d]	p[e]
Progesterone	57-83-0	314.47	*S. obliquus*	0.044	0.99	16	<0.0001
			C. pyrenoidosa	0.018	0.92	39	<0.005
Norgestrel	6533-00-2	312.46	*S. obliquus*	0.017	0.97	40	<0.0001
			C. pyrenoidosa	0.009	0.98	88	<0.0001

[a] The initial concentration of each compound was 1.6 μM in all experiments.
[b] Kinetic rate constant, calculated using the first-order reaction kinetic model ($C = C_0 * \exp(-k * t)$).
[c] Correlation coefficient, which represents the fitness of the modeling data.
[d] Half-life, calculated as (ln 2)/k.
[e] Statistical significance level.

Table 2
Transformation products of progesterone and norgestrel by the two algae.

Product	Name	CAS	Molecular weight	Microalgae	Substrate	Identification method
A	3β-Hydroxy-5α-pregnan-20-one	516-55-2	318	*C. pyrenoidosa*	Progesterone	GC–MS, NIST; Standard match
B	3,20-Allopregnanedione	566-65-4	316	*C. pyrenoidosa*	Progesterone	GC–MS, NIST Standard match
C	1,4-Pregnadiene-3,20-dione	1162-54-5	312	*C. pyrenoidosa* and *S. obliquus*	Progesterone	GC–MS, NIST
D	Androsta-1,4-diene-3,17-dione (ADD)	897-06-3	284	*C. pyrenoidosa* and *S. obliquus*	Progesterone	LC–MS/MS, Standard match
E	17β-Boldenone	846-48-0	286	*C. pyrenoidosa* and *S. obliquus*	Progesterone	LC–MS/MS, Standard match
F	4-Androstene-3,17-dione (AED)	63-05-8	286	*C. pyrenoidosa* and *S. obliquus*	Progesterone	LC–MS/MS, Standard match
G	Testosterone	58-22-0	288	*C. pyrenoidosa* and *S. obliquus*	Progesterone	LC–MS/MS, Standard match
H	5α-Dihydrotestosterone (5α-DHT)	521-18-6	290	*C. pyrenoidosa* and *S. obliquus*	Progesterone	LC–MS/MS, Standard match
I	Epi-androsterone	481-29-8	290	*C. pyrenoidosa* and *S. obliquus*	Progesterone	LC–MS/MS, Standard match
J	4,5-Dihydronorgestrel	20402-55-5	314	*C. pyrenoidosa*	Norgestrel	Derivatization, GC–MS, NIST
K	6,7-Dehydronorgestrel	51087-61-7	310	*C. pyrenoidosa*	Norgestrel	Derivatization, GC–MS, NIST

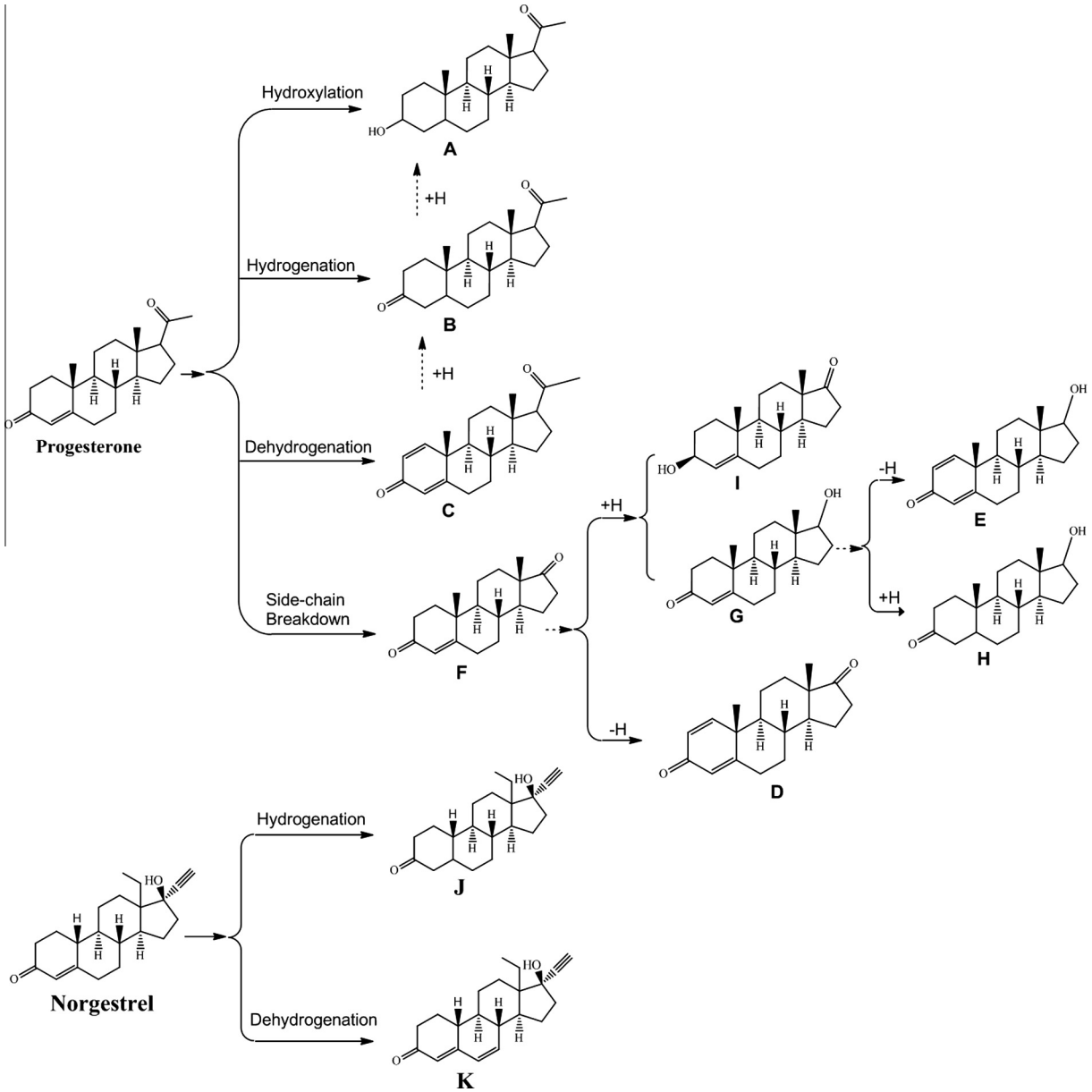

Fig. 3. Tentatively proposed biotransformation pathways for progesterone and norgestrel by *S. obliquus* and *C. pyrenoidosa*. Products **A**–**K** represent the transformation products in Table 2.

et al., 2012). Product **C** has not been reported in algal transformation before, but was detected as a metabolite of progesterone in fungal transformation (Clemons et al., 1989). Conversion of progesterone to some androgens (e.g. AED and ADD) by a bacterium *Mycobacterium smegmatis* has also been reported (Jenkins et al., 2004).

Algal transformation of norgestrel produced only two transformation products 4,5-dihydronorgestrel (**J**) and 6,7-dehydronorgestrel (**K**) due to the hydrogenation and dehydrogenation (Fig. 3). This is the first report on algal transformation of norgestrel and the formation of the two transformation products. However, hydroxyl derivatives have been observed in the degradation of norgestrel by some industrial fungi including *Rhizopus nigricans*, *R. arrhizus* and *Aspergillus niger* (Hu et al., 1996).

In addition to the two progestogens investigated in the present study, the two common freshwater algae species *S. obliquus* and *C. pyrenoidosa* have been known to degrade other organic compounds including alkyl phenols (Zhou et al., 2012) and phthalates (Yan et al., 1995), and to remove metals such as zinc and copper (Zhou et al., 2011). It should be noted that the two algae species are able to grow well in wastewater (Lavoie and De la Noüe, 1985). Therefore, the two algae species could be selected as potential microorganisms for use in the treatment of wastewaters. Further research is required to understand their removal performance in wastewater treatment processes.

The present study also demonstrated that various algae species like *S. obliquus* and *C. pyrenoidosa* could play a significant role in the fate of steroids in the aquatic environment. In fact, microalgae have also shown their ability to degrade or transform other steroids such as hydrocortisone, 4-androsten,3,17-dione, and prednisolone (Abul-Hajj and Qian, 1986; Pollio et al., 1994, 1996; Ghasemi et al., 2008). The present study and those previous studies clearly showed bioconversion between different classes of steroids, which may result in subsequent changes in reproductive endocrine disrupting effects on aquatic organisms. The side-chain breakdown of progesterone could have significant environmental implications due to the generation of androgens. The present study indicates that in addition to the natural excretion from humans and animals, androgens present in the aquatic environment could be produced from the transformation of progesterone by widely present freshwater algae. Some transformation products such as testosterone (**G**), and dehydrotestosterone (**H**) showed in vitro androgenic activity in yeasts and in vivo androgenic effects in fish (Katsiadaki et al., 2002, 2005; Sohoni and Sumpter, 1998). High levels of androgenic activity were measured in the UK surface water and sediment receiving sewage effluent discharge (Thomas et al., 2002). A toxicity identification evaluation study of these samples identified responsible steroid toxicants including dehydrotestosterone (**H**), androstenedione (**F**), androstanedione (**D**), 5β-androstane-3α,11β-diol-17-one, androsterone, and epi-androsterone (**I**). The presence of these androgen compounds at a trace level in rivers may result in masculinization of female wild fish (Jenkins et al., 2001). However, further long-term studies are needed to understand better the fate of these transformation products from algal degradation of progestogens and their potential environmental impacts.

5. Conclusions

The two freshwater algae species *S. obliquus* and *C. pyrenoidosa* were shown to have the capability to transform the two steroids progesterone and norgestrel. Biotransformation was found to be the main mechanism for the removal of these two compounds, while biosorption only played a minor role. Different algal transformation performances were observed between the two algae species, with higher transformation rates found with *S. obliquus*. The synthetic compound norgesterel was found to transform much slower than the natural compound progesterone. Three main transformation products and six minor androgen products were detected for progesterone, while only two products were detected for norgestrel. Hydroxylation, reduction and oxidation as well as side chain breakdown were proposed to be involved in the algal transformation pathways for the two progestogens. The algal transformation of progestogens could have environmental implications in their environmental fate and potential application in wastewater treatment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.10.013.

References

Abul-Hajj, Y.J., Qian, X., 1986. Transformation of steroids by algae. J. Nat. Prod. 49, 244–248.
Chang, H., Wan, Y., Wu, S., Fan, Z., Hu, J., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. Water Res. 45, 732–740.
Clemons, K., Stover, E., Schär, G., Stathis, P., Chan, K., Tökès, L., Stenvens, D.A., Feldman, D., 1989. Steroid metabolism as a mechanism of escape from progesterone-mediated growth inhibition in trichophyton mentagrophytes. J. Biol. Chem. 264, 11186–11192.
Correa-Reyes, G., Viana, M.T., Marquez-Rocha, F.J., Licea, A.F., Ponce, E., Vazquez-Duhalt, R., 2007. Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere 68, 662–670.
DeQuattro, Z.A., Peissig, E.J., Antkiewicz, D.S., Lundgren, E.J., Hedman, C.J., Hemming, J.D.C., Barry, T.P., 2012. Effects of progesterone on reproduction and embryonic development in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 31, 851–856.
Eshrat, G.F., Aroona, C., 2011. Biotransformation of progesterone by pénicillium aurantiogriseum. Res. J Microbiol. 6, 98–104.
Field, C.B., Behrenfeld, M.J., Randerson, J.T., Falkowski, P., 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281, 237–240.
Gao, Q., Tam, N., 2011. Growth, photosynthesis and antioxidant responses of two microalgal species, *Chlorella vulgaris* and *Selenastrum capricornutum*, to nonylphenol stress. Chemosphere 82, 346–354.
Ghasemi, Y., Rasoul-Amini, S., Morowvat, M.H., Mosavi Azam, S., Shokravi, S., Mohagheghzadeh, A., 2008. Bioconversion of hydrocortisone by unicellular microalga Oocystis pusilla. Biotechnology 7, 293–298.
Hirooka, T., Nagase, H., Uchida, K., Hiroshige, Y., Ehara, Y., Nishikawa, J., Nishihara, T., Miyamoto, K., Hirata, Z., 2005. Biodegradation of bisphenol a and disappearance of its estrogenic activity by the green alga Chlorella fusca var. Vacuolata. Environ. Toxicol. Chem. 24, 1896–1901.
Hu, S., Tian, X., Sun, Y., Han, G., 1996. Microbial hydroxylation of 13-ethyl-17β-hydroxy-18, 19-dinor-17α-pregn-4-en-20-yn-3-one. Steroids 61, 407–410.
Hu, S., Tian, X., Han, G., 1998. Novel microbial hydroxylation of 13-ethyl-17β-hydroxy-18, 19-dinor-17α-pregn-4-en-20-yn-3-one. Steroids 63, 88–92.
Huszcza, E., Dmochowska-Gladysz, J., Bartmanska, A., 2005. Transformations of steroids by beauveria bassiana. Z. Naturforsch. C. 60, 103–108.
Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., Moore, R., Nance, M., Brown, A., 2004. Production of androgens by microbial transformation of progesterone in vitro: a model for androgen production in rivers receiving paper mill effluent. Environ. Health Persp. 112, 1508–1511.
Jenkins, R., Angus, R.A., McNatt, H., Howell, W.M., Kemppainen, J.A., Kirk, M., Wilson, E.M., 2001. Identification of androstenedione in a river containing paper mill effluent. Environ. Toxicol. Chem. 20, 1325–1331.
Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.
Katsiadaki, I., Scott, A.P., Mayer, I., 2002. The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. Mar. Environ. Res. 54, 725–728.
Katsiadaki, I., Morris, S., Squires, C., Hurst, M.R., James, J.D., Scott, A.P., 2005. Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive in vivo test for detection of environmental antiandrogens. Environ. Health Persp. 114, 115–121.
Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. PNAS 104, 8897–8901.
Kolpin, D., Furlong, E., Meyer, M., Thurman, E.M., Zaugg, S., Barber, L., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants

in us streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.
Lavoie, A., De la Noüe, J., 1985. Hyperconcentrated cultures of *Scenedesmus obliquus*: a new approach for wastewater biological tertiary treatment? Water Res. 19, 1437–1442.
Li, R., Chen, G.Z., Tam, N.F.Y., Luan, T.G., Shin, P.K.S., Cheung, S., Liu, Y., 2009. Toxicity of bisphenol a and its bioaccumulation and removal by a marine microalga *Stephanodiscus hantzschii*. Ecotox. Environ. Safe. 72, 321–328.
Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment. Pure Appl. Chem. 75, 631–681.
Liu, S., Ying, G.G., Zhao, J.L., Chen, F., Yang, B., Zhou, L.J., Lai, H.J., 2011. Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry. J. Chromatogr. A 1218, 1367–1378.
Liu, S., Ying, G.G., Zhao, J.L., Zhou, L.J., Yang, B., Chen, Z.F., Lai, H.J., 2012. Occurrence and fate of androgens, estrogens, glucocorticoids and progestagens in two different types of municipal wastewater treatment plants. J. Environ. Monit. 14, 482–491.
Majidi, V., Laude Jr., D.A., Holcombe, J.A., 1990. Investigation of the metal-algae binding site with cadmium-113 nuclear magnetic resonance. Environ. Sci. Technol. 24, 1309–1312.
Munoz, R., Guieysse, B., 2006. Algal–bacterial processes for the treatment of hazardous contaminants: a review. Water Res. 40, 2799–2815.
Nakajima, N., Teramoto, T., Kasai, F., Sano, T., Tamaoki, M., Aono, M., Kubo, A., Kamada, H., 2007. Glycosylation of bisphenol a by freshwater microalgae. Chemosphere 69, 934–941.
Pollio, A., Pinto, G., Della Greca, M., de Maio, A., Fiorentino, A., Previtera, L., 1994. Progesterone bioconversion by microalgal cultures. Phytochemistry 37, 1269–1272.
Pollio, A., Pinto, G., Greca, M.D., Fiorentino, A., Previtera, L., 1996. Biotransformations of progesterone by *Chlorella spp*. Phytochemistry 42, 685–688.
Runnalls, T.J., Margiotta-Casaluci, L., Kugathas, S., Sumpter, J.P., 2010. Pharmaceuticals in the aquatic environment: steroids and anti-steroids as high priorities for research. Hum. Ecol. Risk Assess. 16, 1318–1338.
Safiarian, M.S., Faramarzi, M.A., Amini, M., Soltani, N., Tabatabaei-Sameni, M., Hasan-Beikdashti, M., 2012. Microalgal transformation of progesterone by the terrestrial-isolated cyanobacterium Microchaete tenera. J. Appl. Phycol. 24, 777–781.
Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158, 327–339.
Stanier, R., Kunisawa, R., Mandel, M., Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35, 171.
Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydroch. Hydrob. 33, 9–16.
Thomas, K.V., Hurst, M.R., Matthiessen, P., McHugh, M., Smith, A., Waldock, M.J., 2002. An assessment of in vitro androgenic activity and the identification of environmental androgens in United Kingdom estuaries. Environ. Toxicol. Chem. 21, 1456–1461.
Xie, Y.P., Fang, Z.Q., Hou, L.P., Ying, G.G., 2010. Altered development and reproduction in western mosquitofish (*Gambusia affinis*) found in the Hanxi River, southern China. Environ. Toxicol. Chem. 29, 2607–2615.
Yan, H., Ye, C., Yin, C., 1995. Kinetics of phthalate ester biodegradation by *Chlorella pyrenoidosa*. Environ. Toxicol. Chem. 14, 931–938.
Ying, G.G., Kookana, R.S., Ru, Y.J., 2002. Occurrence and fate of hormone steroids in the environment. Environ. Int. 28, 545–551.
Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Lange, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28, 2663–2670.
Zhou, G.J., Peng, F.Q., Zhang, L.J., Ying, G.G., 2011. Biosorption of zinc and copper from aqueous solutions by two freshwater green microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. Environ. Sci. Pollut. R. 19, 2918–2929.
Zhou, G.J., Peng, F.Q., Yang, B., Ying, G.G., 2012. Cellular responses and bioremoval of nonylphenol and octylphenol in the freshwater green microalga *Scenedesmus obliquus*. Ecotox. Environ. Safe. 87, 10–16.
Zucchi, S., Castiglioni, S., Fent, K., 2012. Progestins and antiprogestins affect gene expression in early development in zebrafish (*Danio rerio*) at environmental concentrations. Environ. Sci. Technol. 46, 5183–5192.