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Ecotoxicological effects and removal of 17β-estradiol in *chlorella* algae

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ABSTRACT

17β-estradiol (E2) is a steroid estrogen able to affect the reproduction of aquatic organisms even at extremely low concentrations. The behavior of E2 in the presence of *chlorella* algae was investigated in laboratory experiments. The results showed that the algae's growth was inhibited by 26% after 7 days of culturing in a 2.0 mg L^{-1} solution of E2. The 96 h EC_{50} value of 21.46 mg L^{-1} reflected moderate toxicity. Even low concentrations of E2 were found to affect total chlorophyll and carotenoid levels after 7 and 10 days and to alter stress-generated enzymatic activity in the algae. The efficiency of *chlorella*'s E2 degradation decreased with the increasing of E2 concentration, but 92% of the E2 can be removed from a 0.5 mg L^{-1} solution over 10 days. The degradation mechanism was speculated. The microalgae suffered relatively less growth inhibition at low E2 concentrations, and their removal effectiveness was then better. The data help to elucidate the interaction between *chlorella* algae and E2 in an aquatic environment.

1. Introduction

Steroid estrogens (SEs) can seriously interfere with or even destroy the endocrine systems of human and wild animals due to their effects on reproductive system development, the immune system, the nervous system and much else (Keller et al., 2015). 17β-estradiol (E2) in particular can affect vital activities of aquatic animals even at extremely low concentration (Turdi et al., 2015). For example, male fish can be feminized by exposure to an E2 concentration of less than 1 ng L^{-1} (Susan et al., 1998). Excretion from humans and livestock is the main source of estrogens in nature. It has been reported that E2 is found worldwide in surface water, sludge, sediments, wastewater and landfill leachates (Höhne and Püttmann, 2008). In aquatic environments estrogen content level vary between 0.2 and 580 ng L^{-1} depending on their pollution source (Huang et al., 2013). E2 discharged into aquatic environments affects the growth of bacteria (Lee and Liu, 2002), algae (Chen et al., 2010) and hydrophytes (Pascoe et al., 2002).

Algae are important indicator species for evaluating toxicity in aquatic environments. Previous studies have shown changes in algae's chlorophyll a levels, carotenoids, enzymatic activity and metabolism in the presence of estrogens (Ding et al., 2017). Algae and cyanobacteria have been used to explore the ecotoxicological effects of non-steroidal anti-inflammatory drugs (Bácsi et al., 2016), bactericides (Xiong et al., 2017a) and herbicides (Yan et al., 2012). Chlorophyll a, chlorophyll b and antioxidant enzymes including superoxide dismutase (SOD) in the fresh water alga *Scenedesmus obliquus* have been used to evaluate different toxic effects. Research by Xiong (Xiong et al., 2016) has shown that the total chlorophyll content of *S. obliquus* decreased by 100–200 mg L^{-1} on exposure to carbamazepine compared to controls. There has, however, been little research investigating the ecotoxicological effects of E2 using *chlorella*.

E2 is hard to remove completely in existing wastewater treatment systems and much is discharged directly into aquatic environments. Various processes including bio-degradation (Yu et al., 2007), biotransformation (Teles et al., 2006) and photocatalytic degradation (Mai et al., 2008) reduce the activity of E2 and its residues in the environment. E2 can be removed by *acinetobacter* varieties which have been separated from active sludge, which shows that bacteria make important contributions to estrogen removal (Cai et al., 2016). Algae also play an important role by virtue of their metabolic versatility. *Desmodesmus subspicatus* has been shown capable of removing 75% of 17α-ethinyloestradiol from fresh water (Maes et al., 2014). The E2 removal rate in wastewater could be higher. E2 degradation by algae can reach 30% or more where extracellular peroxidase and humus are present (Li et al., 2017).

Most research in this area has focused on using microalgae to remove high concentrations (several hundred milligrams per liter) of medical pollutants (Wang et al., 2016). There has not yet been in-depth study of the mechanism of E2 degradation by alga-derived organic matter (AOM), nor have ecotoxicological evaluations of microalgae

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been performed.

This study was therefore designed to evaluate toxicity of E2 with respect to *chlorella* species by monitoring their speed of growth and measuring their levels of chlorophyll, carotenoids, and antioxidant enzymes (specifically SODs). Conversely, *chlorella's* ability to degrade E2 was also evaluated. Fluorescence from intracellular organic matter (IOM) and extracellular organic matter (EOM) before and after E2 degradation were analyzed. The changes in levels of proteins, polysaccharides and lipids were also investigated. And the degradation product of E2 was detected. The resulting data provide a basis for algal treatment of estrogen pollution with low-dose toxicity.

2. Materials and methods

2.1. Chemicals

Sodium nitrate, citric acid, ferric ammonium citrate, ethylene diaminetetraacetic acid and chromatographic grade E2 were obtained from Sigma Aldrich. Chromatographic grade acetonitrile, methanol and dichloromethane were supplied by Merck. The highest purity phosphate-buffered saline solution available was purchased from Beyotime. Glacial acetic acid and the other reagents not specifically mentioned were all analytical grade. Milli-Q water from Biogen (electric resistivity > 18 MΩ cm) was used throughout.

2.2. Algae

Chlorella culture methods are shown in the support information.

The algal suspension was diluted using sterilized BG11 medium to achieve an optical density (OD, measured using a using a Shimadzu 2600 UV–visible spectrophotometer) of 1.0 at 680 nm (equal to a dry cell mass concentration of 0.176 g L^{-1} or a cell number count of about 3.75×10^7 cells (determined using a Shineso Algacount F200)). Triplicate algal suspension in various concentrations was used to count cells, weigh dried cell and measure optical densities, resulting finally in a curve relating OD with cell number and OD with dry weight. The dry cell weights were measured using a method published by Gao et al. (2011). Briefly, 10 mL of algal suspension was filtered through dry Whatman (GF/F-44) filter paper and then dried at 105 °C for 24 h. The derived relationships between OD_{680} and the dry weight (g L^{-1}) and OD_{680} and the cell number were

$$\text{Dry cell weight } (\text{g L}^{-1}) = 0.1407 \times OD_{680} + 0.013 \quad (R^2 > 0.99)$$

$$\text{Cell number } (\times 10^7) = 4.0061 \times OD_{680} - 0.1088 \quad (R^2 > 0.99)$$

2.3. Ecotoxicology of 17β-estradiol

A basic growth curve for the algae was obtained by cultivating them for 45 days and testing the suspension's absorbance daily.

2.3.1. Chlorella growth inhibition assay

The detail experimental procedures are in the support information.

2.3.2. Measurement of chlorophyll and carotenoids

The total chlorophyll and carotenoid content were measured using published methods. Cultured cells were harvested from 10 mL of the cell suspension by centrifugation at 10,000 rpm for 10 min using a cooled centrifuge (Allegra 64 R, Beckman Coulter). The supernatant was discarded and the pellet was re-suspended in 10 mL of 99.9% chromatographic grade methanol and incubated in the dark for 24 h at 60 °C. That suspension was then centrifuged again for 10 min at 4500 rpm. The absorbance of the supernatant at 665.2, 652.4 and 470 nm was determined with an Inesa L5S visible spectrophotometer. And the concentrations of c hlorophylla (Chl-a), chlorophyll b (Chl-b) and carotenoids were calculated using the following formulas (Pancha et al., 2015):

$$\text{Chl-a } (\text{mg L}^{-1}) = 16.72A_{665.2} - 9.16A_{652.4}$$

$$\text{Chl-b } (\text{mg L}^{-1}) = 34.09^{-1}A_{652.4} - 15.28A_{665.2}$$

$$\text{Carotenoids } (\text{mg L}^{-1}) = (1000A_{470} - 1.63\ \text{Chl}_a - \text{Chl}_b)/221$$

The absorbance at 470, 652.4 and 665.2 nm were corrected for turbidity by subtracting the absorbance at 750 nm.

2.3.3. Antioxidant enzymes (SOD)

A 50 mL sample of the algal suspension was harvested after 96 h of cultivation and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 0.1 mol L^{-1} phosphate buffer at pH 7.4 and an algae to buffer weight ratio of 1:4. The suspension was sonicated for 5 min at 200 W and a cycle of 5 s of sonication with 10 s intervals in an ice bath to maintain the enzyme's activity. The suspension was then centrifuged again at 10,000 rpm for 10 min at 4 °C. The activity of any antioxidant enzymes in the supernatant was then determined using assay kits supplied by the Nanjing Jiancheng Bioengineering Institute. One-unit antioxidant enzyme activity was defined as the amount that caused a 50% decrease in SOD.

2.4. Removal of E2 by chlorella

One assumption underlying the removal experiments was that algal growth is inhibited at high E2 concentrations. That made careful choice of both the E2 concentration as an environmental stress and of the cell density very important. All the experiments were performed in 500 mL Erlenmeyer flasks. The initial E2 concentrations tested were 1.0, 0.5 and 0.05 mg L^{-1}. The initial algal cell concentration was 0.52 g L^{-1}. The culture conditions were again those described in Section 2.2. All the experiments were carried out in triplicate. More details are in support information.

The percentage biodegradation (P_b) of E2 in the *chlorella* cultures was computed as follows (Xiong et al., 2017b):

$$P_b(\%) = (A_t - A_r - A_d - A_a - A_c)\frac{100}{A_t}$$

Where A_t is the initial concentration of E2 added to the Milli-Q water, A_r is the residual concentration, A_d is the amount of E2 adsorbed, A_a is the E2 removed by abiotically, and A_c is the uptake of E2 by the algae.

Total removal rate of E2=C_t/C_0

C_0 here is the initial E2 concentration at time zero, C_t is the E2 concentration at time t, and k is the degradation rate constant (d^{-1}) and t is time in days.

The bio-concentration factor (BCF) for each treatment was calculated as the ratio of the E2 concentration in the cells ($\mu g_{dry\ weight}$/mL) to the residual E2 concentration (μg/mL) in the medium (Khan and Tansel, 2000).

BCF=(concentration in cells)/(concentration in surrounding medium)

$$BCF = (\mu g_{chemical}/g_{biota})/(\mu g_{chemical}/g_{water})$$

2.5. Extraction of intracellular and extracellular organic matter

The *chlorella* was harvested during their late exponential growth phase. EOM was isolated by centrifuging the cell suspension at 10,000 rpm for 10 min and measuring the content of organic matter in the supernatant. There suspended cells were ultrasonic treatment at low frequency (200 W for 2 h in an ice bath). The fragmented algal cells were centrifuged out at 16,000 rpm over 10 min. The organic matter in the supernatant was taken as the IOM. The intracellular and extracellular supernatants were divided into three portion sand E2 was added at an initial concentration of 1.0, 0.5 or 0.2 mg L^{-1}. The E2 concentration was then measured every 24 h.

2.6. Analysis of 17β-estradiol and its products

E2 was detected by chromatographically (Agilent 1260) (Gu et al., 2016). The detection limit for E2 was determined to be 0.01 mg L^{-1}, and the relative standard deviation for all the triplicate samples was within 5%.

The metabolites after degradation of E2 were extracted using an Oasis HLB column extractor and ethyl acetate (He et al., 2018).

2.7. Polysaccharide, protein and lipid concentrations

The polysaccharide content of the samples (before and after E2 addition) was determined using the anthrone-sulfuric acid method (Sizun et al., 1986). A 1 mL sample of each solution in a brown glass bottle was immersed in an ice bath. After 5 min, 4 mL of anthrone-sulfuric acid solution was added to the bottle. The mixture was cooked for 10 min in a boiling water bath, and then cooled for 10 min with tap water. Finally, the sample was measured at 620 nm with the UV–visible spectrophotometer.

The protein concentrations were analyzed using BCA protein concentration assay kits (Beyotime).

The algae's fat content was measured using the Nile red method. Algal suspensions before and after E2 treatment were centrifuged for 10 min at 10,000 rpm. The supernatant was re-suspended in 20% dimethylsulfoxide and in a water bath temp for 20 min. 1 μL solution of 0.1 mg mL^{-1} Nile red dye was then added. After 5 min the samples were measured at excitation wavelengths of 500–700 nm at 10 nm intervals and an emission wavelength of 575 nm.

Fluorescence spectroscopy, TOC (Total Organic Carbon) and statistical analysis are in the support information.

3. Results and discussion

3.1. Effects of E2 on the growth of chlorella

In Fig. S1, when pH = 5.0 or pH = 9.0, the growth of chlorella was found to be the worst. Compared with the acidic environment, the growth trend of algae was slightly better under weak alkaline conditions. However, the pH of the algae cell was better between 7.0 and 8.0 throughout the culture process, and it grew fastest under neutral conditions. Therefore, the optimum pH value was chosen to be 7.0 ± 0.2. The experiment of different temperature affecting the growth of chlorella is shown in Fig. S2. When the temperature is 15 °C and 40 °C, the growth of chlorella is greatly affected. Optimal growth at 25 °C.

Exposing *chlorella* to E2 concentrations between 1.0 and 2.0 mg L^{-1} showed obvious growth inhibition (Fig. S3). At lower concentrations (0 and 0.5 mg L^{-1}) the growth effects were unclear. The growth inhibition rates were shown in Fig. 1 offers an in-depth explanation. The results also showed that the growth inhibition was increased with the duration of exposure and at high E2 concentrations, however, the growth of microalgae was promoted in 0.05 mg L^{-1} of E2.

Chlorella initially grew slowly as it adapts to its environment. Continuous exposure to E2 slowed growth further. High E2 concentrations damaged the microorganism's cell membranes, which accelerated interaction between E2 and biological substances in the cells. That presumably further impeded growth and may even have caused programmed cell death. *Chlorella* has been reported to have strong resistance to insecticides and anti-bacterial agents, which indicates outstanding adaptability and metabolic flexibility (Kurade et al., 2016). Fig. 1 shows indications of biphasic effects at < 1 mg L^{-1} of E2. Low concentrations of the organic pollutant could promote the microalgae's growth.

Two explanations suggest themselves as to why in these experiments algal growth was restored after 4 days exposure to E2

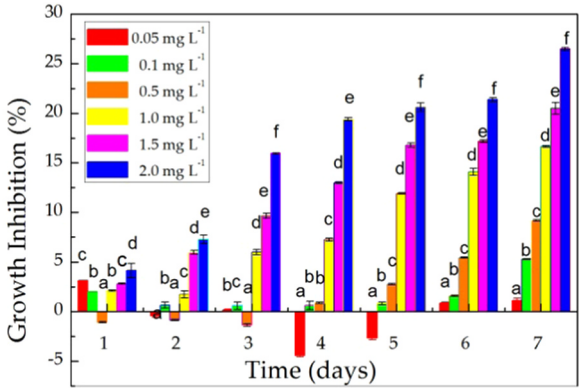

Fig. 1. Inhibition of the growth of *chlorella* algae at different E2 concentrations. The letters above adjacent bars indicate a significant difference ($p \leq 0.05$) between the treatments, whereas the same letter indicates no significant difference.

at < 1 mg L^{-1}. The E2 might have been biodegraded to some less toxic compound. Or perhaps the microalga needs a certain induction period to generate some enzyme indispensable for its metabolism. E2 solution at a concentration of more than 1 mg L^{-1} is known to impede the growth of photoautotrophic aquatic species, though at less than 1 mg L^{-1} it has the opposite effect. Similar results have been observed when *Chlamydomonas reinhardtii* is exposed to certain pesticides (Zhang et al., 2011).

EC_{50} is the concentration at which a substance inhibits growth by 50%. It is a useful index of toxicity. There are two accepted methods of determining chemicals' EC_{50} for algae. One method uses specific growth rate inhibition (Kabra et al., 2014) and the other one uses raw growth rate inhibition (Brezovšek et al., 2014). Some current reports (Xiong et al., 2016) have shown that using raw growth rate inhibition tends to give much lower EC_{50} values. Raw growth rates were used in this study's EC_{50} calculations.

Some results are presented in Table S1. E2 shows relatively high toxicity for *chlorella* over extended exposures. It has an obvious impact even over 96 h. The EC_{50} 21.46 mg L^{-1} is in line with the EC_{50} values from 10.82 to 48.91 mg L^{-1} reported from other studies (Wong and Chang, 1988; Ma et al., 2005; Cáceres et al., 2008). The microalgae react more intensively, which causes a decrease in the EC_{50}, indicating stronger toxicity (Huang et al., 2012).

3.2. Effects of E2 on antioxidant enzymes

Exposure to E2 can lead to an accumulation of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) in the cells. Their strong oxidation potentials may fatally injure the organisms. Excessive ROS can be eliminated or detoxified by antioxidant enzymes such as SOD and CAT to reduce the toxicity (Wang and Freemark, 1995; Zhu et al., 2018). Algae exposed to pollutants often generate SOD in response (Li et al., 2005). SOD activity was tested to further explain the influence of various concentrations of E2 on *chlorella*'s growth.

As is shown in Fig. 2, SOD levels were increased with the increasing of E2 concentration. Comparing cultures at low E2 concentration (0.1 mg L^{-1}) and high E2 concentration (2 mg L^{-1}), the SOD level in the algae was 1.24 times higher 5 days later. By the 10th day, SOD levels had increased significantly ($p \leq 0.05$) at all E2 concentrations compared with the control. Presumably, lipid peroxidation of the cellular membrane and functional damage resulted in the generation of ROS in the cells. SOD was then over-activated in an attempt to convert superoxide free radicals to hydrogen peroxide.

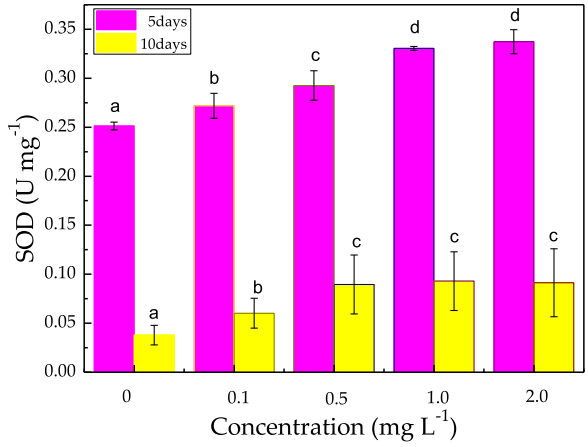

Fig. 2. SOD levels in *chlorella* exposed to different E2 concentrations after 5 days and 10 days. The error bars represent standard deviations (n = 3). Columns with different letters indicate significant differences ($p \leq 0.05$) between control and treatment.

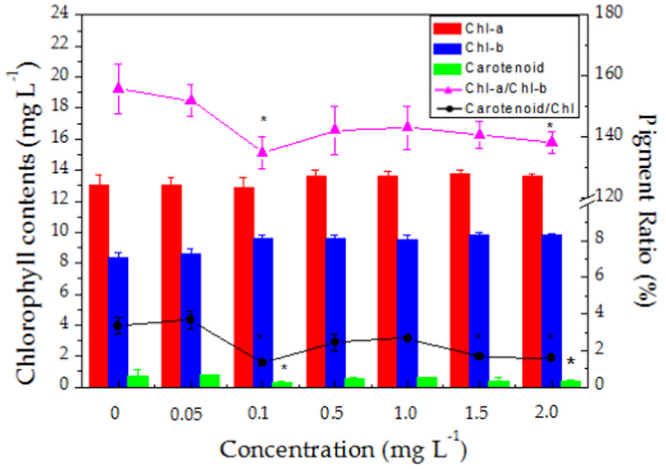

Fig. 3. Effect of E2 on chlorophyll, carotenoid content and pigment ratios in *chlorella* algae after 7 days of exposure. Error bars represent the standard error of the measurements (n = 3). Columns with an asterisk indicate a significant difference ($p \leq 0.05$) between the control and the treatment.

3.3. Chlorophyll and carotenoid content

Chlorophyll a (Chl-a), chlorophyll b (Chl-b) and carotenoid levels were measured to evaluate the impact of E2 on photosynthesis by the algae (Fig. 3). The pigment ratios decreased significantly with E2 concentration ($p \leq 0.01$) at the range of 0.05–0.1 mg L^{-1}.

Photosynthesis is one of the foundational processes by which photosynthetic organisms absorb light and convert light energy into chemical energy. Chlorophyll is a main index of the activity of photosynthetic electron transport. When a plant's cell system is exposed to toxicity, the content of Chl-a can indicate any adverse effects. Reduced Chl-a levels are normally considered a reliable index of pollutant toxicity (Wang and Freemark, 1995).

Chlorophyll levels are also related to the biomass concentration and light penetration. *Chlorella* are exposed to low levels of analgesics, Chl-a and growth are both reduced, probably caused by the intervening compound chlorophyllogen and subsequent chlorophyll transformation. Medicines can thus inhibit the growth of algae (Tsiaka et al., 2013).

In these experiments Chl-a levels were related with carotenoid content, probably because carotenoids can devitalize chlorophyll to eliminate accumulated ROS in chloroplasts (Paliwal et al., 2015). Chl-a can effectively prevent cell damage caused by ROS (Tsiaka et al., 2013). Surprisingly, however, the content of Chl-a at E2 levels > 0.1 mg L^{-1} increased slightly in these experiments, indicating growth promotion at low concentrations. Chl-a in algal cells will increase under oxidative stress. Lipid peroxidation may be one influence. Carotenoids (lutein and carotene) play an important role in light absorption for photosynthesis. The light absorption wavelength ranges of carotenoids and chlorophyll are different, enabling the collection of light energy over a wider wavelength range. In these experiments the content of carotenoid firstly rose slightly with E2 concentration and then decreased. It has been reported in other research carotenoids' oxidation resistance can protect and prevent lipid peroxidation by quenching singlet oxygen and free radicals which are generated in the cells, promoting stability and photosynthetic performance (Jahns and Holzwarth, 2012). Carotenoids could help maintain intact cell membranes as well, essential for cell survival.

The rising ratio of Chl-a/Chl-b and of carotenoids/total Chl indirectly points out that E2 concentration triggers oxidative stress and reduces the activity of PSII (Pal et al., 2011). But since the pollutant concentrations tested were extremely low, stress at the maximum concentration tested had not yet peaked after 7 days compared with the control group. The Chl-a/Chl-b and carotenoids/total Chl ratios also indicate that pigment may have been protected from photo-oxidative damage (Sizun et al., 1986). Park and his colleagues report (Park et al., 2015) that the Chl-a/Chl-b and carotenoids/total Chl ratios of *nannochloropsis* algae were similarly excessive in a high salinity environment.

3.4. Removal of E2 by chlorella

E2 harms *chlorella*, meanwhile, E2 removal by *chlorella* should also be considered. Autotrophy, and carbon fixation and transformation by microalgae to remove organic pollutants are universally recognized (Matamoros et al., 2015).

Over 10 days, no detectable change was observed in the E2 content of the control samples. That suggests non-biological loss was very low. At initial E2 concentrations of 1 and 0.5 mg L^{-1}, overall removal efficiencies by *chlorella* of 65% and 92% respectively were observed after 10 days. Such efficiencies then decreased with increasing E2 concentration (Fig. S4).

Bio-absorption, bio-accumulation and bio-degradation are three E2

Table 1
E2 removal by *chlorella* algae through bioadsorption, bioaccumulation and biodegradation over 10 days.

Initial E2 concentration (mg·L^{-1})	Mass balance of E2 removal (%)					E2 degradation kinetics	
	Bioadsorption	Bioaccumulation	Biodegradation	Abiotic removal	Total removal (%)	k (h^{-1})	R^2
0.05	20.6 ± 0.7	8.3 ± 0.3	69.9 ± 1.7	–	98.8 ± 0.5	ND	ND
0.5	17.6 ± 0.2	13.1 ± 0.2	61.4 ± 0.8	–	92.1 ± 0.5	0.35	0.70
1.0	27.1 ± 1.3	16.5 ± 0.6	21.8 ± 0.4	–	65.4 ± 0.3	0.11	0.83

ND- not detected. The E2 concentration was below the chromatograph's detection limit.
k- removal rate constant (d^{-1}).
R^2- correlation coefficient.

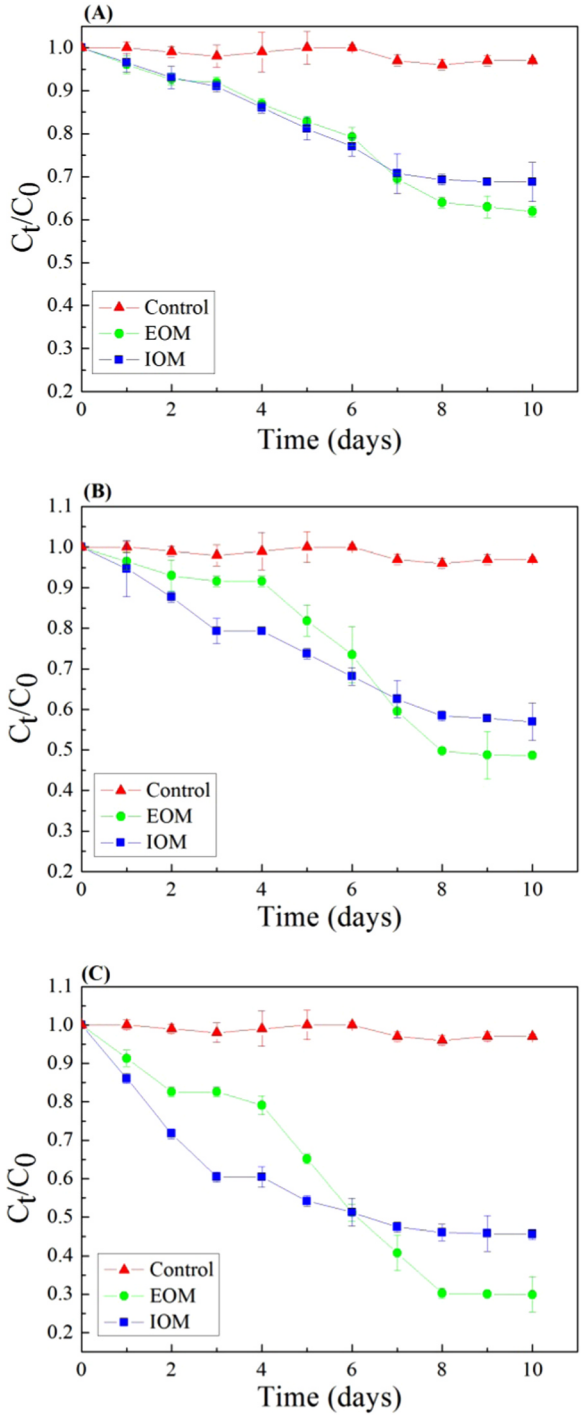

Fig. 4. The biodegradation of E2 at concentrations of 1.0 mg L^{-1} (A), 0.5 mg L^{-1} (B) and 0.2 mg L^{-1} (C) in the presence of EOM and IOM with an initial pH of 7.0. Error bars represent the standard deviation of three replicates.

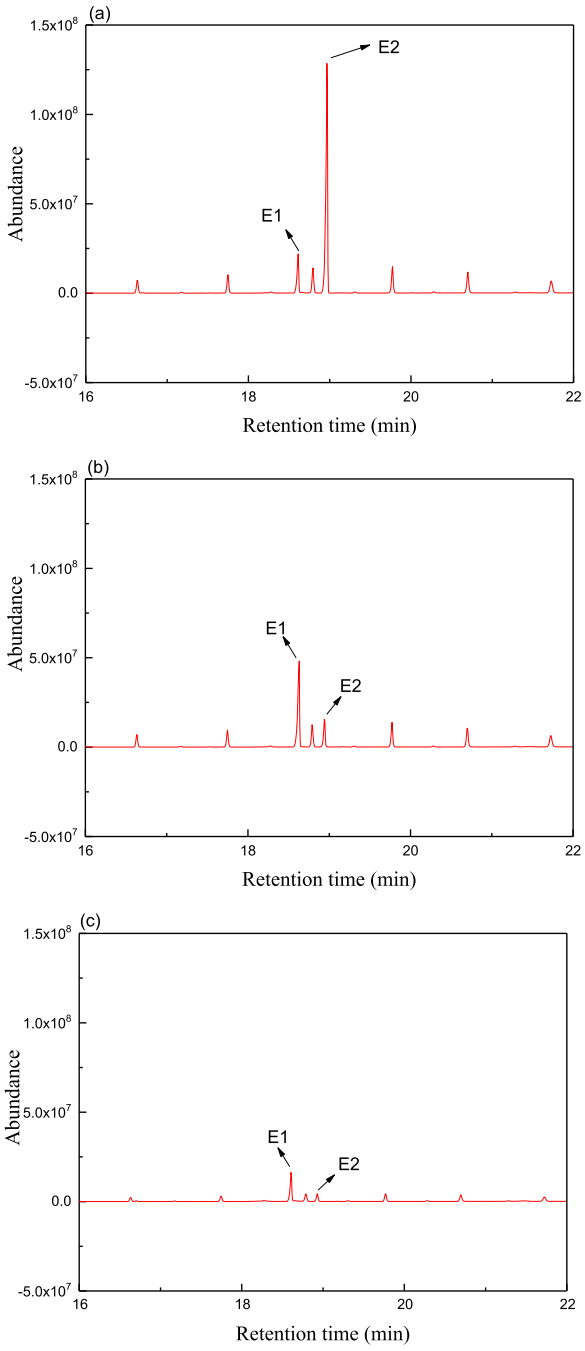

Fig. 5. GC-MS spectra of E2 and its main biodegradation products on day 3 (a), day 7(b) and day 10 (c).

removal processes in biological systems. Bioaccumulation in aquatic organisms is a basic process of pollutant dissipation in aquatic environments. Fig. S5 shows that after being cultured for 5 and 10 days in E2 solution, the BCF values at 0.05 mg L^{-1} of E2 were obviously higher than that at 0.5 or 1.0 mg L^{-1}. With prolonged exposure the BCF values increase and peak at 22.4 at the 10th day with 0.05 mg L^{-1} of E2. At higher E2 concentrations the BCF values are much lower because the algae absorb E2 and it remained concentration decreased in such batch experiments.

E2 that adhered to the cell walls cannot be ignored either. It reached 20.6% in the 0.05 mg L^{-1} solution and was 17.6% at 0.5 mg L^{-1} and 27.1% at 1.0 mg L^{-1} of E2. Table 1 presents those data. The average total biodegradation after 10 days with 0.05, 0.5 and 1.0 mg L^{-1} of E2 was 69.9%, 61.4% and 21.8% respectively (Table 1). Higher E2 concentration produced less percentage removal. First-order kinetic was used to explain the degradation. The calculated degradation rate constants (k_s) of E2 at 1 mg L^{-1} and at 0.5 mg L^{-1} were 0.11 and 0.35 respectively.

3.5. Algal-derived organics in E2 degradation

Previous research reports that AOM promotes diuron's photodegradation (Li et al., 2015). It can be assumed that AOM played a considerable role in the degradation of E2 in the presence of algae.

Metabolic activity removed a portion of the E2. Degradation of the remainder may also then can be mediated by polysaccharides, proteins and lipids released from the algae.

Fig. 4 shows E2 biodegradation in the presence of EOM and IOM. The removal of E2 decreases with increasing of E2 concentration in the presence of EOM or IOM, which is in accordance with the data presented in Fig. S4. In the 0.2 mg L^{-1} E2 solution, 70% and 54% of the E2 were eventually removed over 10 days with EOM and IOM present, respectively. The E2 removal at all concentrations was better in the presence of EOM rather than IOM. The concentration of E2 in the blank controls remained unchanged.

Fluorescence spectroscopy was used to characterize the AOM. The supernatants were scanned for fluorescence after removal of any *chlorella* cells, which means that some of the organic substances bound to the cytoderm gave no fluorescence signal. Moreover, polysaccharides do not fluoresce (Villacorte et al., 2015). The assay therefore detected mostly proteins, humic substances and fulvic acid. The three-dimensional fluorescence spectrum of *chlorella* (Fig. S6) has two peaks were identified with EOM, the strongest appearing at an Ex/Em of 280/310. As is shown in Table S2, the peak strength at 89.3 corresponds to some soluble microbial by-product (Chen et al., 2003). The second peak (strength at 15.5) was observed at an Ex/Em of 320/405 representing humic acids. That is consistent with the results reported by Villacorte (Villacorte et al., 2015). The IOM and EOM peaks are in a same region (Ex/Em of 280 nm/310 nm) but the fluorescence intensity of IOM is larger (strength at 214.2). The results show that the IOM contained more soluble microbial products (Ex/Em of 280/310 nm) than the EOM, mostly high molecular weight substances such as tyrosine-like and tryptophan-like proteins. Another IOM peak was the aromatic protein region with an Ex/Em of 330/225 nm. All the fluorescence intensities had decreased after 10 days of incubation with 1.0 mg·L^{-1} of E2. That suggests that both IOM and EOM contribute to E2 degradation.

Table S3 shows a calculated percentage removal for each EOM and IOM component over ten days. The removal of intracellular protein (20.5%) was higher than that of extracellular (11.1%), while the decrease in extracellular polysaccharides (15.5%) was slightly higher than for intracellular (11.2%). Amino acids are degraded by proteins, especially tyrosine-like proteins, during E2 degradation. That plays an important role in the degradation. So, biodegradation of AOM plays some part in the removal of E2 by *chlorella* algae. Many previous studies have found (Robbins et al., 1980) that tryptophan components contained in water soluble extracellular polymeric substances can be converted to singlet states by irradiation, and that tryptophan cation transfer to triplet state radicals, leading to the degradation of E2.

3.6. E2 degradation products

GC/MS analysis shows that chlorella can convert E2 to a less active estrogen E1 during biodegradation, and that eventually the levels of both substances decreased. Some data are shown in Fig. 5. E1 is not the final product of E2 biodegradation. It may be further decomposed by the algae into other unknown substances. The process is complicated. Some of the degradation products may be volatile organic compounds difficult to detect by these methods.

4. Conclusions

The data show that at less than 1 mg L^{-1}, concentration of E2's inhibition of *chlorella*'s growth is negligible. Its growth inhibition depends on concentration and time. A decrease in photosynthetic pigments in *chlorella* and good SOD activity indicate that the algae can protect itself from oxidative injury to some extent. When used for bioremediation of E2, *chlorella* shows better removal and bio-accumulation at low E2 concentrations. Proteins contribute most to the degradation of E2 by AOM, and E1 is an intermediate in the biodegradation. *Chlorella* species are a potential candidate for bioremediation of E2-contaminated natural waters.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.01.129.

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