Oxidation mechanism and overall removal rates of endocrine disrupting chemicals by aquatic plants

A.R. Reis^a, K. Tabei^b, and Y. Sakakibara^b

^a Department of Soil Science, Federal University of Lavras, Lavras-MG 37200-000, Brazil.

^b Department of Civil and Environmental Engineering, Waseda University, 169-8555, 3-4-1 Okubo, Shinjuku, Tokyo, Japan. *E-mail: andrereis@dcsl.ufla.br ; sakaki@waseda.jp

Abstract

The purpose of this study was to evaluate experimentally and theoretically the oxidation mechanisms and overall removal rates of phenolic endocrine disrupting chemicals (EDCs) by aquatic plants. EDCs used in this study were bisphenol-A (BPA), 2,4-dichlorophenol (2,4-DCP), 4-tert-octylphenol (4-t-OP), and pentachlorophenol (PCP). Referring to reported detection levels in aquatic environments and contaminated sites, the feed concentration of each EDC was set from 1 to 100 µg/L. Experimental results showed that, except for PCP, phenolic EDCs were stably and concurrently removed by different types of aquatic plants over 70 days in long-term continuous treatments. Primal enzymes responsible for oxidation of BPA, 2,4-DCP, and 4-t-OP were peroxidases (POs). Moreover, enzymatic removal rates of BPA, 2,4-DCP, and 4-t-OP by POs were more than 2 orders of magnitude larger than those by aquatic plants. Assuming that overall removal rates of EDCs are controlled by mass transfer rates onto liquid films on the surface of aquatic plants, an electrochemical method based on the limiting current theory was developed to measure the mass transfer rates of EDCs. Because of extremely large removal rates of EDCs by POs, observed removal rates by aquatic plants were in reasonably good agreement with calculated results by a mathematical model developed based on an assumption that mass transfer limitation is a rate-limiting step.

Keywords: Endocrine disrupting chemicals; hydrogen peroxide; peroxidase; biological Fenton reaction, phytoremediation

1. Introduction

Environmental pollutants such as EDCs have been detected in aquatic environment, and their effects on human health and aquatic life forms have received considerable attention [1]. Especially, the estrogenic effects of BPA, 4-t-OP, and estrogens such as estrone (E1), 17 β -estradiol (E2), and 17 α -ethinylestradiol (EE2) were identified for aquatic lives [1, 2]. In addition, there are suspected EDCs such as PCP and 2,4-DCP with potential estrogenic activities. Concentration ranges of these EDCs were reported as 0.5 to 21 μ g/L for BPA in stream/river [3], 5 ng/L to 4 μ g/L for 4-t-OP in rivers [4], 26 ng/L to 20 μ g/L for 2,4-DCP in rivers [5], and 2 to 82 ng/L for natural and synthetic estrogens in effluents from sewage treatment plants [6]. Higher levels of EDCs up to 7 and 17 mg/L for phenols and BPA were found in leachates of waste landfills, respectively [7, 8]. Because of identified and suspected endocrine disrupting effects, effective methods for removal of these EDCs from contaminated sites are required.

Former studies on EDCs removal demonstrated the possibility of EDCs treatments by plants, plant tissues, and plant enzymes. Table 1 shows an example of these studies, demonstrating that EDCs such as BPA, 2,4-DCP, 4-t-OP, NP, E1, E2, and EE2 could be removed by enzymes such as peroxidases (POs) [9, 10, 11, 19], laccase (LAC) [12, 13], polyphenol oxidase (PPO) [10, 14], and glutathione S-transferase (GST) [15] as well as plant cells or tissues containing enzymes [16, 17, 18]. In addition, some researchers [9, 18] confirmed that treatment of phenolic EDCs by plant enzymes or plant tissues was effective to eliminate estrogenic activity from contaminated sites. As shown in Table 1, most studies on EDCs treatment have been conducted based on batch experiments with relatively high concentrations of EDCs (i.e. mg/L level) when compared with observed levels in aquatic environments. Batch experiments could indicate the possibility of removing EDCs, however, continuous performance and/or stability have not been well known. Furthermore, studies focusing on mechanisms and kinetics of EDCs removals have not been conducted extensively; and therefore, rational operation and design conditions with information on appropriate plants have not been available.

This study focused on oxidation mechanisms and removal rates of trace EDCs by aquatic plants. Continuous treatments of BPA, 2,4-DCP, 4-t-OP and PCP were conducted using different aquatic plants, and the stability and removal rates were

investigated. In our previous study [19], EDCs concentration was set continuously at 100 µg/L. However, in this study, the feed concentration of BPA, 2,4-DCP, 4-t-OP and PCP was set in the range of 1 to 100 µg/L to investigate the removal ability of aquatic plants at different influent concentration. In addition, enzymatic removal rates of EDCs were measured to elucidate primal enzymes and reactions responsible for the EDCs removal in phytoremediation process. The same measurements and comparison were made for removals of estrogens such as E1, E2, and EE2. To better understand the overall removal rates by aquatic plants, an electrochemical method based on the limiting current theory was developed to measure the mass transfer rates of EDCs. A mathematical model was developed based on an assumption that overall removal rates of EDCs are controlled by the mass transfer rates in liquid film onto the surface of aquatic plants, and comparisons of calculated and observed results were made to evaluate the net removal rates of EDCs by aquatic plants.

2. Materials and methods

2.1. Aquatic plants

Aquatic plants used in this study were floating plants, Amazon frogbit (*Limnobium laevigatum*), Giant Duckweed (*Spirodela polyrhiza*), Duckweed (*Lemna aoukikusa*), Liver wort (*Ricciocarpos natans*), and Crystalwort (*Riccia fluitans*); submerged plants, Willow Moss (*Fontinalis antipyretica*), Hydrilla (*Hydrilla verticillata*), Pondweed (*Potamogeton oxyphyllus*), Hornwort (*Ceratophyllum demersum*); and an emergent plant, Watercress (*Nasturtium officinale*).

Enzyme activities in aquatic plant tissues were measured according to previously studies [13, 33, 34] and were listed in Table 2 with parameters such as specific surface area (a), endogenous H_2O_2 level, and water content. Enzymatic activities were converted to number of units (U) based on extinction coefficient such as 26.6 mM/cm for POs, 1.42 mM/cm for PPOs, 3.6 mM/cm for LAC, and 9.6 mM/cm for GST. One unit of enzyme activity (U) represents the amount of enzyme catalyzing 1 µmol of a reference substrate per 1 min in the corresponding enzymatic reaction.

2.2. Continuous treatments of EDCs by aquatic plants

Referring to reported detection levels in contaminated sites, the feed concentration of BPA, 2,4-DCP, 4-t-OP and PCP was set in the range of 1 to 100 µg/L. The stock solutions of each EDC were prepared and dissolved in a diluted Hoagland solution.

Continuous treatments of BPA, 2,4-DCP, 4-t-OP and PCP were conducted using an experimental apparatus illustrated in Fig. 1, where each plant was cultivated in glass vessels (effective liquid volume was 5 L). All vessels were maintained under intermittent illumination, which consisted of light and dark periods of 16 h and 8 h, respectively. The illumination during the light period was controlled at 3000 lux and was provided by white fluorescent lamps. The flow rate of the feed solution was set at 1.92 L/day (corresponding to HRT = 2.6 days) using peristaltic pumps.

Every experiment and analysis was conducted at a room temperature (25°C). In continuous experiments, the amount of aquatic plant was set at 5 g-FW (fresh weight)/L and the feed solution was prepared every 5 days. The fresh biomass of aquatic plants was monitored and the excess over than 5 g-FW/L was removed. In parallel to the continuous experiments, a blank test without any aquatic plant was conducted as a reference. After the end of the continuous treatment, plants were taken out from the vessels and the amount of EDCs accumulated in their tissues was analyzed.

To investigate the possible effect of microorganisms on EDCs degradation, part of aquatic plants were collected from the vessels after 35 days of operation, and were disinfected with sodium hypochlorite (5%) for 10 min as described by Toyama et al. [13], and afterwards, washed with distilled water several times to avoid physiological damage. After that, batch treatments of BPA, 2,4-DCP, 4-t-OP and PCP were conducted and compared with those by undisinfected aquatic plants.

2.3. Removal rates of EDCs by extracted crude enzymes

In order to determine the primal enzymes in the EDC treatments, different fractions of crude POs were extracted from homogenized aquatic plants so as to conduct further *in vitro* batch treatments of phenolic EDCs. The following extraction steps were carried out at 4°C. For each enzyme, the aquatic plant tissues were homogenized in a buffer solution (buffer volume (mL):fresh weight (g) = 1:1) using a mortar with a pestle. The PO

fractions, soluble PO (SPO), ionically cell wall-bound PO (IPO), and covalently cell wall-bound PO (CPO) were extracted at pH 6.0 in 50 mM tris-maleate, 0.2 M CaCl_2 , and 40 mM tris-maleate buffer as described in a previous study [34]. The homogenate was centrifuged at $10000\times g$ for 30 min, and the supernatant was used for the measurement of enzyme activities as well as for the *in vitro* treatment of EDCs. In order to simulate the degradation of phenolic EDCs by enzymes in plant cells, each crude enzyme fraction of POs, PPO, LAC and GST was extracted according to previous studies [13, 33, 34]. To avoid any interference, *in vitro* treatments of phenolic EDCs were performed with a buffer for each enzyme. In this study, all enzymatic degradation reactions were performed in a capped erlenmeyer flask. Initial concentration of each phenolic compound (BPA, 2,4-DCP, 4t-OP, PCP, E1, E2 and EE2) was individual set at 100 $\mu\text{g/L}$, and 1 mL of each crude enzyme was added separately in 40 mM tris-maleate (pH 6.0) and 0.15 mM H_2O_2 . The final volume was completed to 100 mL and the enzymatic reactions, performed at 25°C , were stopped after 3 h. EDCs concentrations were measured in triplicate and a control experiment without crude enzyme was conducted as reference.

2.4. Measurements of mass transfer rates

The mass transfer rates of EDCs were experimentally measured and analyzed using an electrochemical technique based on the limiting current theory proposed in our previous study [35]. The experimental apparatus was set at the same conditions as described in continuous experiment. The surface of aquatic plant was sprayed with an electromagnetic paint (MAC-115 spray, Yugen-Mac Corporation). After the paint dried, oxidation-reduction potential (ORP) and pH probes were installed in the vessel, and the potential and current applied were monitored using a multimeter. The experiment was carried out using an electrolyte solution of 10 mM copper sulfate and 50 mM sulfuric acid diluted in deionized water.

The electric current was increased in small increments until a well-defined decrease in the ORP was observed. At this point, the limiting current was reached [35]; and the thickness of liquid film (δ) and the mass transfer coefficient of EDCi (k_{Li}) could be analyzed by the follow equations:

$$\delta = \frac{2F D_{Cu^{2+}} C_{Cu^{2+}}}{i_{lim}} \quad (1)$$

$$k_{Li} = \frac{D_{EDCi}}{\delta} \quad (2)$$

Where i_{lim} is the limiting current density (A/cm²), F is Faraday constant (9.648x10⁴ (C/mol)), $D_{Cu^{2+}}$ is the molecular diffusion coefficient of Cu²⁺ (cm²/s), $C_{Cu^{2+}}$ is the bulk concentration of Cu²⁺ (i.e. 10⁻⁵ mol/cm³ in this study), and D_{EDCi} is the molecular diffusion coefficient of component i of EDCs. The diffusion coefficient of Cu²⁺ used to calculate the thickness is 0.72x10⁻⁵ cm²/s [36]. Molecular diffusion coefficients of EDCs were estimated using Wilke and Chang relationship [37] as follows: (cm²/day) BPA = 0.195; 2,4-DCP = 0.281; 4-t-OP = 0.189; PCP = 0.226; E1 = 0.178; E2 = 0.211; and EE2 = 0.167.

2.5. Chemical analysis of phenolic EDCs and estrogens

Chemical analysis of EDCs and estrogens were referred to tentative investigation manual [38]. In the analysis of aqueous solutions of BPA, 2,4-DCP, 4-t-OP, and PCP, 100 mL of water samples were extracted two times with 10 mL of dichloromethane 5000 (Wako Pure Chemical Industries, Ltd.), after adding 5 g of NaCl, 0.2 mL of 1 M HCl solution, and 5 µL of surrogate. The surrogate was spiked to confirm the analytical recoveries and precision. The extracted samples were then transferred to capped erlenmeyer flasks, and 3 g of sodium sulfate was added and kept for over 12h. Thereafter, trimethylsilyl (TMS) derivatives were obtained and were measured using GC/MS (Shimadzu QP5050A).

The standards of E1, E2, EE2, and 17β-estradiol (16,16,17-d₃) were purchased from Wako Chemicals (Japan). The internal standard 17β-estradiol (16,16,17-d₃) was spiked in water samples to confirm analytical recoveries and precision. In solid phase extraction of the estrogens, C18 cartridges (Sep-Pak Plus, Waters) were conditioned sequentially with 5 mL of acetone, 5 mL of methanol, 5 mL of pure water (3 times), water samples, 5 mL of pure water, and 5 mL of hexane. The working solution was extracted using 5 mL

of ethyl acetate/acetone (1:1/v:v) through the cartridges. The final sample extracts were transferred to autosampler vials and evaporated to dryness with a gentle stream of nitrogen. After the addition of derivatives such as 5 µL of naphthalene, 25 µL of pyridine and BSTFA + 1% TMCs for E1 and E2 analysis, and 130 µL of pyridine and BSTFA + 1% TMCs for EE2, the vials were heated at 70°C for 30 minutes. The silylated derivatives obtained were analyzed by GC-MS.

Phenolic EDCs accumulated in aquatic plant tissues were also measured. The procedure extractions of EDCs were carried out after the following pretreatments. First, the aquatic plants were washed with distilled water, submerged in liquid nitrogen, and ground in methanol using a mortar and a pestle. After that, the EDCs were extracted with dichloromethane, and the analysis was performed in the same procedure as water samples.

2.6. Endogenous H_2O_2

The endogenous H_2O_2 concentration in aquatic plants was measured according to Okuda et al. [39]. Aquatic plant samples (0.3 g-FW) were ground in liquid nitrogen using a mortar and pestle, and the finely powdered material obtained was mixed with 1 mL of 0.2 M perchloric acid. The mixture was centrifuged at $12000 \times g$ and 4°C for 15 min. Afterwards, by adding horseradish PO (Wako Pure Chemicals) and prescribed chemicals, H_2O_2 concentration was measured at 590 nm using a standard calibration curve. The endogenous H_2O_2 concentration was expressed in µmol/kg-FW.

2.7. Histochemical localization of POs and H_2O_2

Transverse sections of the stem of *C. demersum* were obtained using a freehand microtome. After that, the histochemical localization of POs was conducted by placing a sample in 40 mM tris maleate buffer containing 84 mM guaiacol and 3 mM H_2O_2 . The enzymatic reaction for staining the sample was carried out in 1 min, where PO activity in plant cell structures was recorded as a brown color formation. From the stained sample, we found that POs were located in almost every part of aquatic plants; therefore, another sample was also stained with guaiacol for the localization of endogenous H_2O_2 . As reference, treatments without H_2O_2 and guaiacol were conducted and their results

were compared with the stained samples. The sections of the samples were washed with distilled water and were observed using a microscope (Olympus BX60).

3. Results and discussion

3.1. Continuous treatments

Figs. 2 (A)-(D) show experimental results of continuous treatments of BPA, 2,4-DCP, 4-t-OP and PCP by aquatic plants such as *N. officinale*, *C. demersum*, and *R. natans*. Influent concentration of each EDC was changed in a stepwise manner from 10 µg/L to 1, 100, and 10 µg/L to evaluate the ability of aquatic plants to remove BPA, 2,4-DCP, 4-t-OP and PCP at different influent concentrations. No removals of EDCs were observed in blank experiments (no aquatic plants). During the first 44 days, effluent concentrations of BPA, 2,4-DCP, and 4-t-OP declined quickly, **approaching** steady-state values. Similar results were obtained using different aquatic plants for long-term continuous treatments of EDCs in our previous studies [19, 25]. These results demonstrated that very stable and concurrent removals of EDCs except PCP were achieved by aquatic plants.

During days 45 and 56, the effluent concentrations of BPA and 4-t-OP from the vessel of *C. demersum* became larger than the influent concentration of 1 µg/L; however, in the subsequent treatment the concentrations became lower than the influent concentration (100 µg/L) during days 57 and 65. From these results, it could be seen that when influent concentration became very low, longer periods of treatment would be required to reach steady state conditions. After day 66, the influent concentration was returned to 10 µg/L and the effluent concentrations of most EDCs became similar **to** levels observed during the first 44 days. Regarding the treatment of PCP, no significant removal was observed in any vessels of aquatic plants. This compound was used as a pesticide and its persistency and toxicity were reported in the literature [40]. Therefore, it is considered that PCP is too refractory to be decomposed or oxidized by aquatic plants or enzymes.

To evaluate the possible influence of microorganisms associated with aquatic plants, batch treatments of BPA, 2,4-DCP, 4-t-OP and PCP by disinfected plants (*L. laevigatum* and *S. polyrhiza*) were conducted and compared with those by undisinfected plants. As shown in Figs. 3 (A) to (D), no significant differences on BPA, 2,4-DCP, 4-t-OP, and

PCP removals were observed between disinfected and undisinfected plants. In a comparison using *S. polyrhiza*, Hoang et al. [41] also reported no significant effect of the plant-bacteria association on the removals of BPA, 2,4-DCP and NP. From these results, it was thought that continuous and stable removals of BPA, 2,4-DCP, and 4-t-OP were achieved by aquatic plants as shown in Figs. 2 (A)-(C).

After 70 days of operation in the continuous experiments, aquatic plants were taken out and amounts of EDCs accumulated in plant tissues were analyzed. However, the amounts were less than 5% of total EDCs removed in the experiment (data not shown). Referring to former studies shown in Table 1, it is considered that except for PCP, the EDCs removed were oxidized or degraded by certain enzymes in aquatic plants.

To evaluate removal performances of EDCs by different aquatic plants, the specific removal rates based on the wet surface areas of aquatic plants were calculated by:

$$r_{surface} = \frac{(\Delta EDC_{plant} - \Delta EDC_{blank})}{a \times X \times HRT} \quad (3)$$

Where,

$r_{surface}$ = specific removal rate of EDCs based on the wet surface area of aquatic plant ($\mu\text{g}/\text{cm}^2/\text{day}$),

ΔEDC_{plant} = difference of EDC concentrations between influent and effluent at steady state condition in vessel of aquatic plant ($\mu\text{g}/\text{L}$),

ΔEDC_{blank} = difference of EDC concentrations between influent and effluent of blank vessel at steady state condition ($\mu\text{g}/\text{L}$),

HRT = hydraulic retention time (day),

X = biomass concentration (g-FW/L),

a = specific surface area of aquatic plant ($\text{cm}^2/\text{g-FW}$).

Observed removal rates of BPA, 2,4-DCP, 4-t-OP and PCP are shown against effluent concentration (C_{EDC}) in Fig. 4, where the removal rates at influent concentrations of 10 and 100 $\mu\text{g}/\text{L}$ in this study, as well as results observed in our previous study [19]

were also plotted. Data at an influent concentration of 1 $\mu\text{g/L}$ was not adopted, because it was considered that the steady state condition was not reached.

As shown in Fig. 4, except for PCP the specific removal rates of EDCs, r_{surface} were in the range of 0.02 to 0.66 ($\mu\text{g}/\text{cm}^2/\text{day}$) and were similar regardless of different species or morphologies of aquatic plants. The specific removal rate based on biomass of aquatic plants can be obtained by multiplying the specific surface area (a) of aquatic plant to r_{surface} defined by Equation (3). Corresponding specific removal rates ranged from 0.54 to 31 ($\mu\text{g}/\text{g-FW}/\text{day}$). Furthermore, the specific removal rates tended to increase with increasing bulk or effluent concentration of EDCs. The removal rates of PCP (plotted by $\blacktriangle$ *N. officinale*, $\circ$ *C. demersum*, and $\bullet$ *R. natans*) in continuous treatments were very low and 1 or 2 orders of magnitude smaller than those of BPA, 2,4-DCP, and 4-t-OP. This happens because aquatic plants show no ability to remove PCP from aqueous solutions.

3.2. *In vitro* phenolic EDCs treatment by extracted crude enzymes

In order to evaluate the mechanism or primal enzymes responsible for the EDCs removals, batch treatments of BPA, 2,4-DCP, 4-t-OP, and PCP, as well as estrogens such as E1, E2, and EE2 were conducted using crude enzymes extracted from aquatic plants. Table 3 shows observed removal efficiencies, where treatment or reaction times for POs and other enzymes (i.e. PPO, GST, and LAC) were set at 3 and 12 h, respectively. Regardless of the reaction times, relatively higher removal efficiencies of BPA, 2,4-DCP, 4-t-OP, E1, E2 and EE2 were observed for POs, while poor efficiencies for PPO, GST, and LAC. Similar results for *in vitro* phenolic EDCs treatments by crude enzyme extracted from different aquatic plants were observed in our previous study [19]. The removal efficiency by POs varied depending on EDCs and estrogens. For example, estrogens such as E1, E2 and EE2 were removed 100% by POs. It is interesting to note that total removal efficiencies of most EDCs except PCP by POs were close to or more than 100%. Comparing Tables 2 and 3, we found that plants with higher POs activities, such as *C. demersum* and *R. fluitans*, showed higher removal efficiencies in the enzymatic *in vitro* treatments.

Regarding PCP, removal efficiencies were relatively smaller in comparison with other EDCs. In addition, in the cases of IPO and CPO, relatively larger efficiencies up to 50% were observed. The reason for these removals was not known, but as no significant removal of PCP was observed in continuous treatments (Fig. 2), we suppose that the removals were mainly attributable to adsorption to some constituents in extracts. This is supported by the work of Song and Huang [42], who reported that PCP was removed from about 30% of its initial concentration by sorption processes in a batch treatment using *L. polyrhiza*.

The results mentioned above indicated that primal enzymes responsible for the treatments of BPA, 2,4-DCP, and 4-t-OP, E1, E2, and EE2 were POs that catalyze the following oxidation reaction:

According to Equation (4), endogenous H_2O_2 concentration in aquatic plants is a key substance for EDCs degradation inside plants. During oxidation processes, phenolic compounds were converted catalytically to phenoxy radicals, which polymerize EDCs and then formed less soluble products in cell or water [9, 10]. On the other hand, regarding the final products of EDCs, a study [14] reported that BPA was degraded to CO_2 and H_2O via two phenolic intermediates by *Caragana chamlagu*, while different products such as carbohydrate conjugates [11], glycosylation products [43], polymer and 4-isopropenylphenol [44], and hydroxylation products [11] were reported by different researches using different plant species, plant tissue or plant enzyme. Further studies will be needed to identify final products formed inside aquatic plants.

To analyze the relations between the removal rates of EDCs by aquatic plants (Fig. 4) and by crude enzymes (Table 3), the specific removal rates by PO enzymes were calculated by:

$$r_{\text{enzyme}} = \frac{(\Delta EDC_{\text{plant}} - \Delta EDC_{\text{blank}})}{a \times X^* \times \Delta T} \quad (5)$$

Where, X^* is a corresponding biomass concentration of aquatic plant (10 g-FW/L in this study) from which crude enzymes (i.e. POs) were extracted, and ΔT is the treatment time (3 h in the case of POs).

The maximum values of the specific rates of each EDC and estrogen calculated by Equation (5) were in the range of 6 to 70 $\mu\text{g}/\text{cm}^2/\text{day}$, which were almost 2 orders of magnitude larger than the overall removal rates by aquatic plants. This indicates that the oxidation of EDCs and estrogens by POs was not the rate-limiting step.

3.3. Histochemical localization of POs and endogenous H_2O_2

In the histochemical localization using guaiacol and H_2O_2 , it is possible to visualize the location of POs in plant cells. The results for *C. demersum* are shown in Figs. 5 (A) to (C). Looking at the sections of stem (C) treated with guaiacol and H_2O_2 , we found that almost every part of the stem cells possesses POs. In addition, highly concentrated zones of POs were observed in epidermis and cell walls. Similar results regarding the localization of POs in epidermis and vascular tissues for *Vicia faba* roots were reported by Jensen [45].

Fig. 5 (A) indicates the control section made without guaiacol/ H_2O_2 treatments. As shown in (B), the oxidation of guaiacol was observed mainly in cell walls or adjacent areas, indicating relatively higher concentration zones of endogenous H_2O_2 . Wang et al. [46] reported a similar result that histochemical localization of H_2O_2 in wheat plants was observed mainly in cell wall structure. It is considered that the results on the localizations of POs and H_2O_2 indicate the oxidation sites of EDCs by peroxidases. In addition, Fig. 6 illustrates the oxidation mechanisms proposed for EDCs degradation. It is known that endogenous H_2O_2 is produced through photochemical or respiration reaction in mitochondria [47]. Endogenous H_2O_2 is utilized for the oxidation of most phenolic EDCs catalyzed mainly by POs or biological Fenton reaction [19]. PCP is not oxidized by POs; however, in the presence of adequate concentrations of Fe^{2+} , this compound is quickly oxidized by biological Fenton reaction through the consumption of endogenous H_2O_2 in aquatic plants [19, 25]. In our recent study [48], the OH radical formation via biological

Fenton reaction in aquatic plants was experimentally confirmed by electron spin resonance analysis.

3.5 Mass transfer rates and comparisons with a mass transfer model

Assuming that overall removal rates of EDCs are controlled by mass transfer rates in liquid films, the specific removal rates defined by Equation (3) or the flux of EDCs can be calculated by:

$$J_{EDCi} = k_{Li} C_{EDCi} \quad (6)$$

Where, J_{EDCi} is bulk or effluent concentration of component i of EDCs.

Limiting current density of analyzed aquatic plants were 0.325×10^{-3} (A/cm²) for *S. polyrhiza*, 0.431×10^{-3} (A/cm²) for *L. laevigatum*, 0.56×10^{-3} (A/cm²) for *C. demersum* and 0.712×10^{-3} (A/cm²) for *N. officinale*, respectively. Thickness of liquid film analyzed by Equation (1) where an adjustment of the film thickness due to migration effect, according to the analytical procedure [35]. The liquid film thickness (δ) of analyzed aquatic plants were 195 μ m for *N. officinale*, 248 μ m for *C. demersum*, 322 μ m for *L. laevigatum*, and 427 μ m for *S. polyrhiza*, respectively. Therefore, the mass transfer coefficient, k_{Li} in Equation (6) can be estimated using molecular diffusion coefficient of EDCs (D_{EDCi}) and analyzed liquid film thickness (δ) of aquatic plants.

Theoretically calculated results of J_{EDCi} are shown in Fig. 4 by solid lines in comparison with long-term continuous observed results. In the calculation, δ values were assumed to be the average value of analyzed aquatic plants (around 300 μ m in this study), while calculated fluxes for the smallest δ (195 μ m) and the largest (427 μ m) are shown by dashed and dotted lines. Calculated results were in reasonably good agreement with observed results of BPA, 2,4-DCP, and 4-t-OP by aquatic plants (Fig. 4). On the other hand, plots for removal rates of PCP were displaced from theoretically calculated results as shown in Fig. 4(D). This happens because different types of aquatic plants show no capability to remove PCP from aqueous solutions (Fig. 2(D)),

showing extremely low removal rates. Therefore, a more powerful oxidant reaction is needed to oxidize PCP from contaminated sites.

Fig. 7 shows the same comparisons of specific removal rates of E1, E2, and EE2. Observed specific rates were obtained from batch experiments (data not shown). Calculated removal rates for estrogens agreed well with observed results. From the comparisons in Figs. 4 and 7, it can be concluded that overall removal rates of EDCs and estrogens by aquatic plants were controlled by mass transfer rates in liquid film, because enzymatic reaction rates by POs were 1 or 2 orders of magnitude larger than the mass transfer rates in liquid film.

4. Conclusions

In continuous experiments, except for PCP, EDCs such as BPA, 2,4-DCP, and 4-t-OP were stably and concurrently removed by aquatic plants over 70 days. No significant effect of aquatic plant-bacteria association on the EDCs removal was observed.

POs were primal enzymes responsible for the BPA, 2,4-DCP, 4-t-OP, E1, E2, and EE2 removals. POs were located in almost every part of stem cells, while higher activities were found in cell walls of aquatic plants.

Specific removal rates of BPA, 2,4-DCP, 4-t-OP, E1, E2, and EE2 by POs were more than 2 orders of magnitude larger than those by aquatic plants.

Continuous long-term removal rates of BPA, 2,4-DCP and 4-t-OP by aquatic plants were in reasonably good agreement with theoretically calculated results developed based on an assumption that mass transfer in liquid film is a rate-limiting step.

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Table 1. Former studies on EDCs treatments using plants, cells or plant enzymes.

Plants or cells	EDCs*	Concentration	Enzyme*	Mode	Reference
<i>Eichhornia crassipes</i>	PCP	500 µg/L	GST	Batch	[4]
Soybean	BPA, 2,4-DCP	100-200 mg/L	POs	Batch	[9]
Crude enzyme from potato	BPA	120 mg/L	PPO	Batch	[10]
Plant suspension cell	BPA	1 mg/L	-	Batch	[11]
<i>Nicotiana tabacum</i>	BPA	10 mg/L	-	Batch	[12]
<i>Caragana chamlega</i>	BPA	97 mg/L	-	Batch	[14]
<i>Ipomea aquatica</i>	BPA	5 mg/L	-	Batch	[16]
<i>Arabidopsis thaliana</i>	2,4,6-trichlorophenol	2-8 mg/L	LAC	Batch	[12]
<i>Spirodela polyrhiza</i>	2,4-DCP, phenol	5 mg/L	LAC	Batch	[13]
Floating and submerged aquatic plants	BPA, 4-t-OP, NP	10 µg/L	-	Batch and Continuous	[14]
<i>Portulaca oleracea</i>	BPA, OP, NP	5-114 mg/L	-	Batch	[18]
Soybean	BPA	2.5 mg/L	POs	Batch	[22]
<i>Lemma gibba</i>	E1, E2, EE2	1-40 µg/L	-	Batch and Continuous	[23]
<i>Portulaca oleracea</i>	BPA, OP, NP, E2	7-13 mg/L	POs	Batch	[24]
Floating and submerged aquatic plants	BPA, 4-t-OP, 2,4-DCP	100 µg/L	POs	Continuous	[19]
Floating and submerged aquatic plants	BPA, 4-t-OP, 2,4-DCP	100 µg/L	-	Continuous	[25]
Fruits and vegetables	BPA	100 mg/L	PPO	Batch	[26]
<i>Portulaca oleracea</i>	BPA	1.2 mg/L	PPO	Batch	[27]
Radish, perennial ryegrass	BPA	4.6-46 mg/L	-	Batch	[28]
Floricultural Salvia plants	BPA	1.2 mg/L	-	Batch	[29]
<i>Bruguiera gymnorhiza</i>	BPA	0.5-60 mg/L	-	Batch	[30]
<i>Phragmites australis</i>	E1, E2, EE2	6 ng/L	-	Continuous	[31]
Radish, ryegrass	NP	1 mg/L	-	Batch	[32]

*Abbreviations of EDCs and enzymes are as follows:

EDCs - Endocrine disrupting chemicals, PCP - pentachlorophenol, BPA - bisphenol A, 2,4-DCP - 2,4-dichlorophenol, 4-t-OP - 4-tert-octylphenol, NP - nonylphenol, E1 - estrone, E2 - 17β-estradiol, EE2 - 17α-ethinylestradiol, POs - peroxidases, PPO - polyphenol oxidase, LAC - plant laccase, GST - glutathione S-transferase.

Table 2. Specific surface area, water content and enzyme activities in aquatic plants.

Plant species	<i>a</i>	Water content	SPO	IPO	CPO	PPO	GST	LAC	H ₂ O ₂
	cm ² /g-FW	(%)	U/g-FW	U/g-FW	U/g-FW	U/g-FW	U/g-FW	U/g-FW	μmol/kg-FW
<i>C. demersum</i>	19.6	94.3	103	27.5	35.3	1.94	35.3	0.39	99.1
<i>R. fluitans</i>	18.6	95.1	26.7	9.51	3.80	2.05	1.17	4.84	149
<i>L. laevigatum</i>	24.5	94.8	13.9	7.10	4.34	3.41	0.50	1.12	611
<i>S. polyrhiza</i>	47.4	93.6	9.51	2.34	0.13	1.67	4.19	1.13	301
<i>H. verticillata</i>	21.3	94.4	6.06	0.47	0.21	2.43	1.69	0.84	365
<i>F. antipyrretica</i>	36.8	97.1	14.2	6.17	2.96	3.28	0.55	0.64	105
<i>N. officinale</i>	17.9	95.7	6.22	7.18	0.91	1.44	0.70	1.18	459
<i>P. oxyphyllus</i>	15.6	90.7	16.2	14.1	1.26	3.68	1.46	1.94	114
<i>R. natans</i>	27.2	94.9	8.48	11.6	4.43	3.19	13.5	0.36	534

*Abbreviations of enzymes are as follows:

a: Specific surface area of plants, SPO: soluble peroxidase, IPO: ionically cell wall-bound peroxidase, CPO: covalently cell wall-bound peroxidase, PPO: polyphenol oxidase, GST: glutathione S-transferase, LAC: laccase, H₂O₂: hydrogen peroxide

Table 3. Removals of phenolic EDCs and estrogens by different crude enzymes.

Crude enzymes+	Plant Species	Removal efficiency (%)						
		BPA	2,4-DCP	4-t-OP	PCP	E1	E2	EE2
SPO*	<i>C. demersum</i>	99.2	80.9	98.1	14.2	100	100	100
	<i>R. fluitans</i>	99.5	99.2	99.3	5.80	100	100	100
	<i>L. laevigatum</i>	88.5	26.2	76.6	8.22	82.6	95.5	100
	<i>S. polyrhiza</i>	98.5	35.3	87.8	24.4	65.2	95.7	100
IPO*	<i>C. demersum</i>	99.1	69.5	95.6	26.2	100	100	100
	<i>R. fluitans</i>	96.9	76.1	94.9	50.8	81.2	87.3	100
	<i>L. laevigatum</i>	4.79	57.3	30.1	36.1	89.4	83.6	86.1
	<i>S. polyrhiza</i>	65.8	40.1	59.4	30.3	87.4	95.7	93.2
CPO*	<i>C. demersum</i>	90.6	52.9	78.9	34.7	100	100	100
	<i>R. fluitans</i>	97.6	75.1	96.6	46.9	84.2	95.7	100
	<i>L. laevigatum</i>	2.24	38.2	24.8	50.7	38.9	32.9	69.9
	<i>S. polyrhiza</i>	38.6	51.7	44.8	52.1	59.8	81.3	87.4
PPO**	<i>C. demersum</i>	4.06	47.4	31.1	30.1	8.23	3.37	13.3
	<i>R. fluitans</i>	1.19	55.3	37.4	32.1	18.4	22.6	15.6
	<i>L. laevigatum</i>	89.9	44.9	79.8	26.6	2.68	29.4	29.9
	<i>S. polyrhiza</i>	93.4	35.9	70.2	25.1	28.6	56.3	83.4
GST**	<i>C. demersum</i>	16.2	19.6	18.6	8.48	1.32	9.75	3.39
	<i>R. fluitans</i>	9.99	5.55	2.80	2.05	1.43	12.7	2.38
	<i>L. laevigatum</i>	8.43	10.4	7.40	2.63	0.24	2.72	4.06
	<i>S. polyrhiza</i>	13.2	6.27	3.84	7.52	0.87	1.24	16.1
LAC***	<i>C. demersum</i>	10.4	8.86	10.6	5.38	0	20.5	0.14
	<i>R. fluitans</i>	15.5	19.9	20.6	11.4	0.12	31.1	31.1
	<i>L. laevigatum</i>	7.15	4.83	10.2	7.08	2.77	26.4	43.1
	<i>S. polyrhiza</i>	10.1	7.59	11.9	5.84	25.9	60.1	80.9

Abbreviations of EDCs are as follows:

BPA - bisphenol A, 2,4-DCP - 2,4-dichlorophenol, 4-t-OP - 4-tert-octylphenol, PCP - pentachlorophenol, E1 - estrone, E2 - 17 β -estradiol, EE2 - 17 α -ethinylestradiol,

+ Crude enzyme concentrations correspond to 10 g-FW/L

* Reaction time and H₂O₂ concentration were set at 3h and 0.15 mM, respectively

** Reaction time was set at 12h

*** Reaction time and dissolved oxygen concentration were set at 12h and 10 mg/L, respectively

Fig. 1. Scheme of experimental apparatus.

Fig. 2. Continuous treatments of (A) bisphenol-A; (B) 2,4-dichlorophenol, (C) 4-t-octylphenol, and (D) pentachlorophenol by aquatic plants.

▲ *S. polyrhiza* (disinfected) △ *S. polyrhiza* (undisinfected)
 ◆ *L. aoukikusa* (disinfected) ◇ *L. aoukikusa* (undisinfected)

Fig. 3. Batch treatments of (A) bisphenol-A, (B) 2,4-dichlorophenol, (C) 4t-octylphenol and (D) pentachlorophenol by disinfected and undisinfected aquatic plants.

▲ *N. officinale* ○ *C. demersum* ● *R. natans* (This study)
 □ *C. demersum* ◇ *F. antypiretica* ◆ *L. laevigatum* ■ *L. aoukikusa* △ *S. polyrhiza* [25]
 — δ (300 μm) - - δ (195 μm) - · - δ (427 μm)

Fig. 4. Comparison of observed and calculated results for removal rates of (A) bisphenol A, (B) 2,4-dichlorophenol, (C) 4-t-octylphenol and (D) pentachlorophenol.

Fig. 5. Photomicrographs showing the localization of POs in the root with and without guaiacol/H₂O₂ treatment: (A) stem of untreated *C. demersum*; (B) stem of *C. demersum* treated with guaiacol; (C) stem of *C. demersum* treated with guaiacol/H₂O₂.

Fig. 6. A proposed mechanism for oxidations of EDCs and PCP in aquatic plants.

$\diamond$ *R. fluitans* $\square$ *S. polyrhiza* $\circ$ *C. demersum* $\triangle$ *L. laevigatum*
 — δ (300 μm) - · - δ (195 μm) - - δ (427 μm)

Fig. 7. Comparison of observed and calculated removal rates of (A) estrone, (B) 17 β -estradiol and (C) 17 α -ethinylestradiol.

Graphical Abstract

A proposed mechanism for oxidations of endocrine disrupting chemicals and persistent pollutants such as pentachlorophenol in aquatic plants.