Removal of Aqueous 17 α -Ethinylestradiol by *Rhodococcus* Species

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Received: August 21, 2008

Accepted in revised form: June 18, 2009

Abstract

Pharmaceutical substances such as 17 α -ethinylestradiol (EE2), the active component of the contraceptive pill, are often not eliminated during wastewater treatment and are persistent in the environment. Using solid-phase extraction and chromatography methods for analyzing low concentrations of EE2 and metabolites, biodegradability of EE2 was studied using four bacteria grown under aerobic conditions in the absence or presence of a readily available carbon source (2.5 g/L of adipic acid or glucose). Preliminary identification of produced metabolites was also completed. In presence of a cosubstrate, *Rhodococcus erythropolis* removed EE2 up to 47% of its initial concentration (1.4 and 0.5 mg/L) in 13 h, while it removed only 10% after 75 h without a cosubstrate. In the presence of a cosubstrate, *Rhodococcus equi* removed EE2 up to 39% of its initial concentration (1.4 mg/L) after 65 h, whereas no significant EE2 removal was observed using *Rhodococcus rhodochrous* and *Rhodococcus zopfii*. Preliminary analysis of EE2 transformation products confirmed the presence of phenol and a compound of high molecular weight (331 amu). These results improve our understanding of the effect of common biological wastewater treatment variables such as bacteria strains, acclimation, and presence of cosubstrate, on the removal of EE2.

Key words: 17 α -ethinylestradiol; *Rhodococcus erythropolis*; *Rhodococcus equi*; *Rhodococcus zopfii*; *Rhodococcus rhodochrous*

Introduction

SEVERAL STUDIES HAVE SHOWN THAT natural and synthetic estrogens are often not eliminated during wastewater treatment, and additionally are not biodegraded in the environment (Ternes *et al.*, 1999b; Heberer, 2002; Jurgens *et al.*, 2002; Yoshimoto *et al.*, 2004). 17 α -Ethinylestradiol (EE2), a synthetic estrogen used in the oral contraceptive pill, has been detected in ecologically relevant amounts in sewage effluents, surface water, river water, bed sediments, and in activated and digested sludge (Weber *et al.*, 2005). In 10 Canadian municipal wastewater effluents (MWWs), EE2 was discharged into the environment at concentrations of up to 42 ng/L (Ternes *et al.*, 1999b), with a median concentration of 9 ng/L. The maximum concentration of EE2 from four MWWs in Michigan was measured to be 0.8 ng/L (Parrott and Blunt, 2005). Baronti *et al.* (2000) measured EE2 concentrations up to 13 ng/L in untreated sewages in the Tiber River in Rome, Italy. Ternes *et al.* (1999b) also measured estrogen levels in 16 German municipal sewage treatment effluents and found concentrations of EE2 up to 15 ng/L. Last, is that

EE2 was detected in groundwater and in raw and purified drinking water in Germany up to concentrations of 2.4 ng/L (Heberer, 2002). In fish, as in all other vertebrates, estrogens play an important role in many reproductive and developmental processes. For example, less than 1 ng/L EE2 and even as little as 0.1 ng/L can stimulate male rainbow trout to produce an egg yolk protein called vitellogenin normally only associated with sexually mature female (Jurgens *et al.*, 2002); and 4 ng/L caused male fathead minnows failure to develop normal secondary sexual characteristics such as gonad development, sex determination, and reproductive maturity (Jurgens *et al.*, 2002). *In vivo* tests have demonstrated that EE2 is approximately 11–27 times more potent than the natural female sex hormone 17 β -estradiol (E₂) (Huber *et al.*, 2004), suggesting that EE2 is one of the most important endocrine disruptors found in the environment.

The presence of estrogens, detected wherever sought, and the identification of their negative impact on aquatic systems stimulated research on the fate of EE2 during wastewater treatment. The removal of estrogens in activated sludge treatment has been investigated in previous studies. Urase and Kikuta (2005) examined the removal of E₂, estrone (E₁), and EE2, among other pharmaceuticals, by activated sludge with batch experiments using a lab-scale reactor. The EE2 concentration remained relatively stable and exhibited the highest adsorption rate (as high as 60% of the initial concentration after 20 min), whereas the majority of the

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pharmaceuticals had low adsorption tendencies (Urase and Kikuta, 2005). The authors concluded that EE2's removal from treatment plants was more a function of excess sludge extraction than actual degradation. Aerobic batch experiments were conducted by Ternes *et al.* (1999a) to determine the biodegradability of natural and synthetic estrogens using a slurry of activated sludge from a sewage treatment plant (STP) that was diluted in drinking water. Estrogens were spiked to levels of 0.01 and 1 mg/L. Approximately 20% of the EE2 at a concentration of 0.01 mg/L was removed after 24 h (Ternes *et al.*, 1999a). Further confirming the persistence of EE2 in activated sludge, mestanol was removed by 80% after 24 h, producing EE2 as a metabolite, which in turn, showed no signs of removal even after 72 h. A study performed by Weber *et al.* (2005) supported the results obtained by Ternes *et al.* (1999a). Using activated sludge from a membrane bioreactor and from a conventional STP, EE2 remained persistent after 70 h (Weber *et al.*, 2005). Shi *et al.* (2004) and Vader *et al.* (2000) demonstrated that EE2 could be removed using nitrifying activated sludge (NAS). Approximately 90% of EE2 was removed after 96 h (Shi *et al.*, 2004). An unknown transient degradation intermediate of EE2 was produced. NAS was also shown to remove EE2 within 6 days in a study where activated sludge was enriched with nitrifying microorganisms (Vader *et al.*, 2000).

Few studies have focused on the removal of estrogens, specifically EE2 using pure cultures. Shi *et al.* (2002) isolated *Fusarium proliferatum* from a cowshed sample and identified it as a micro-organism capable of removing EE2. With an initial high concentration of 25 mg/L, 97% of EE2 was removed in 15 days (Shi *et al.*, 2002). Degradation products were observed by reverse-phase high-performance liquid-chromatography (RP-HPLC) analysis; however, they were not identified. An ammonia-oxidizing bacterium, *Nitrosomonas europaea*, was able to remove natural and synthetic estrogens during a long sample period of 187 h (Shi *et al.*, 2004). Unidentified degradation products were observed from an EE2 sample after 120 h. Yoshimoto *et al.* (2004) identified four strains of microorganisms from an enrichment culture of activated sludge from wastewater treatment plants capable of removing E₁, E₂, estriol (E₃), and EE2. Of the four strains, three were of *Rhodococcus equi*, Y50155, Y50156, and Y50157 and one was *Rhodococcus zopfii*, Y50158. The removal of EE2 was studied using an initial concentration of 100 mg/L. Each *R. equi* strain removed EE2 by 70% in 8 h and by 80% in 24 h (Yoshimoto *et al.*, 2004). *R. zopfii* was particularly efficient in degrading all four estrogens demonstrating a removal of 70% in 6 h for EE2 (Yoshimoto *et al.*, 2004). After 24 h, all four estrogens were completely removed. No degradation products were determined by gas chromatography-mass spectrometry (GC-MS) analysis.

In each of the aforementioned studies, when degradation products were produced and detected they were not identified. Also common to the studies reviewed was the lack of an easily degraded carbon source during the removal of EE2. The objective of the work presented here was to investigate the removal of EE2 in the presence of an easily degraded carbon source and to determine the presence of EE2 metabolites. Accordingly, the bacteria *Rhodococcus zopfii*, *Rhodococcus rhodochrous*, *Rhodococcus equi*, and *Rhodococcus erythropolis* were selected in an aim to identify microorganisms capable of removing EE2, to determine the effect of the presence of a co-

substrate and to study the nature of some of the metabolites produced during the removal of EE2.

Materials and Methods

Chemicals

EE2 (CAS #57-63-6) and E2 (CAS #50-28-2, used as a surrogate) were purchased from Sigma-Aldrich (Oakville, Canada) with purities of 98%. All bacteria, *Rhodococcus equi* (ATCC #13557), *Rhodococcus erythropolis* (ATCC #4277), *Rhodococcus zopfii* (ATCC #51349), and *Rhodococcus rhodochrous* (ATCC #13808) were purchased from the American Type Culture Centre (ATCC, Rockville, MD). EE2 solutions were prepared in HPLC-grade water. Nutrients were purchased from Sigma-Aldrich. Culture media were purchased from Becton Dickinson (Oakville, Canada).

EE2 removal experiments

R. rhodochrous and *R. equi* were first cultivated aerobically in culture media flasks containing Brain Heart Infusion (BD 237500) and Nutrient Broth (BD 234000), respectively. *R. erythropolis* and *R. zopfii* were cultivated aerobically in culture media flasks containing Pancreatic Digest of Casein and Yeast Extract (BD 211705 and 212750). All flasks were kept in an incubator shaker operating at 150 rpm and 26°C.

Using absorbance analysis, bacterial growth was monitored at 500 nm with a UV-visible spectrophotometer (Varian Cary 100 Bio, Mississauga, Canada) after sample centrifugation and dilution. Once the bacteria had reached the exponential phase in the culture medium, it was transferred to a generic growth medium referred to as Modified Mineral Salt Media (MMSM) [NH₄NO₃, 4 g/L; KH₂PO₄, 4 g/L; Na₂HPO₄, 6 g/L; MgSO₄ · 7H₂O, 0.2 g/L; CaCl₂ · 2H₂O, 0.01 g/L; FeSO₄ · 7H₂O, 0.01 g/L; Na₂EDTA, 0.014 g/L]. A cosubstrate known to be easily metabolized by the selected bacteria was added to the MMSM (except for one set of experiments with *R. erythropolis* performed without a cosubstrate). Based on optimal growth conditions defined in our previous work, adipic acid was added as a cosubstrate for *R. erythropolis*, *R. zopfii*, and *R. rhodochrous* at a concentration of 2.5 g/L of MMSM, whereas glucose was used as a cosubstrate for *R. equi* at 2.5 g/L. Yeast extract was also added at a concentration of 0.1 g/L. In the case where adipic acid was used as the cosubstrate, the pH of the MMSM was adjusted to approximately 7.0 using 1.0 N sodium hydroxide solution before autoclaving.

After bacteria were acclimatized to MMSM, 10 mL of bacteria entering the exponential growth phase were used to inoculate the 500-mL sterilized Erlenmeyer flasks containing appropriate cosubstrate (except for the set of experiments with *R. erythropolis* performed without cosubstrate). Unless stated otherwise, each set of experiments involved 11 flasks. Table 1 provides the details of the conditions used in the shake flasks to investigate the removal of EE2. The three high and three low flasks were used to study the removal of EE2 at two different concentration levels (except for experiments involving *R. equi*, as described in Table 1). The concentrations used, 1.4 and 0.5 mg/L, were selected in the low range of concentration used in previous biodegradation studies (0.01 to 100 mg/L). These concentrations, higher than the concentrations found in wastewater effluents (up to 50 ng/L) al-

TABLE 1. EXPERIMENTAL FLASKS CULTURED UNDER AEROBIC CONDITIONS

Flasks	Number of flasks		EE2 concentration (mg/L)	Conditions
	Other micro-organisms	R. equi		
High concentration	3	2	1.4	Active bacteria
Low concentration	3	0	0.5	Active bacteria
Control—adsorption	3	1	1.4 or 0.5	Dead biomass
Control—abiotic degradation	2	1	1.4	No biomass

EE2, 17 α -ethinylestradiol.

lowed for the monitoring of EE2 concentration and facilitated the recovery of the degradation products for identification. When the bacteria reached stationary phase, 100 mL of EE2 solution was added to each flask. The resulting total solution volume was 200 mL in each experimental flask. Samples (40 mL) were collected from each flask to measure the exact initial concentration of EE2. Flasks were covered with a foam top to maintain the aerobic conditions and returned to the incubator shaker. Further samples were taken at time intervals over 24 to 80 h. Each sample collected was centrifuged and the supernatant collected was acidified using a few drops of 5.0 M HCl to lower the pH below 3.0 such that biological activity would cease. The supernatant was then placed in 40-mL glass amber vials (Fisher Scientific, Ottawa, Canada) and stored in the fridge at 4°C for no more than 72 h prior to preparation for analysis.

Due to findings reported by Urase and Kikuta (2005), three adsorption control flasks were also used to measure the contribution of adsorption on biomass in reducing the EE2 concentration (see Table 1). The abiotic degradation control flasks were for observing any EE2 removal naturally occurring under the experimental conditions. These control flasks were sterilized to cease bacterial growth prior to EE2 addition.

Collection of volatile organic compounds

An experimental procedure was designed for the collection of any volatile organic compounds (VOCs) that were released during the experiments. Only *R. erythropolis* was selected for this experiment given the favorable results obtained by *R. erythropolis* for EE2 removal (see Results). *R. erythropolis* was cultivated in one 500-mL Erlenmeyer flask in the same manner as previously described. EE2 was added during the stationary phase at an initial concentration of 1.4 mg/L. The flask was placed in the incubator shaker and two plastic tubes were inserted into the foam top of the flask. One tube supplied a very low flow of nitrogen to the flask, in order to transport the VOCs through the second tube to a collection tube placed inside the incubator. The collection tube was packed with silanized glass wool and 1 g of silicon oxide Chromosorb WHP (Supelco, Inc., lot #R23484, Oakville, Canada). Due to the foam top and low flow of nitrogen, aerobic conditions were maintained. To recover the volatile products in the collection tube, the silicon oxide beads were transferred to a headspace vial. The vial was set in an ice-water bath at 0°C while the oxidizing environment of the vial was removed and replaced with helium. The vial was removed from the water bath and brought to room temperature. Using a DigiPrep

Jr. graphite heating block coupled with a built-in thermocouple, the vial was heated to 120°C and remained at that temperature for 1 h. A 10- μ L aliquot of the headspace was injected into the GC-MS.

Analytical Methods

Solid-phase extraction

Bond Elut C18 cartridges (Varian) were placed on a SPE manifold system (Supelco, Inc., Visiprep™) and were conditioned sequentially with 6 mL acetone, 6 mL ethanol, and 6 mL HPLC-grade water adjusted to pH 7.5. Ten milliliters of a 5-mg/L E₂ solution (21.5% ethanol/HPLC water), acting as the surrogate, were added to the 40-mL samples. Samples were filtered using 1- μ m glass fiber filters (Fisher Scientific). The pH of the samples was adjusted to pH 7.5 (or higher), and they were then pumped through the cartridges at a rate of approximately 10 mL/min using a vacuum pump (BioRad™, Mississauga, Canada). Once the extraction was completed, the cartridges were rinsed with 10 mL of pH 7.5 HPLC-grade water. The cartridges were dried for 1 min. The analytes were eluted twice with 3 mL of ethanol. The ethanol extracts were completely evaporated under a gentle flow of nitrogen. Four milliliters of HPLC-grade water and 1 mL of ethanol was added to reconstitute the sample for HPLC analysis. The final sample was filtered using a 13-mm syringe filter (PVDF, 0.22 μ m, Fisher Scientific) and stored in 2.0 mL-amber HPLC vials. Based on the performance surrogate, analyte recoveries ranged from 94–100%.

HPLC analysis

The concentration of EE2 was measured using an Agilent 1100 Series HPLC equipped with a Zorbax SB-C18 column (3.5 mm, 4.6 $\times$ 75 mm) and guard cartridge SB-C18 (5 mm, 4.6 $\times$ 12.5 mm). HPLC operating conditions and parameters were as follows: mobile phase: A = 20 mM NaH₂PO₄, pH 2.0 (pH adjusted with H₂SO₄), B = acetonitrile; 1.0 mL/min flow rate; 50% acetonitrile, 50% 20 mM NaH₂PO₄ gradient; UV detector, 279 nm; 28°C; 10-min stop time; 10 μ L injection volume. The limit of detection (LOD) was determined to be 0.58 mg/L, which yielded a limit of analysis (LOA) of 1.73 mg/L (three times the LOD). Taking into account the preconcentration of the samples by solid-phase extraction, the overall LOD and LOA were 0.073 and 0.22 mg/L, respectively. The precision of the method was evaluated at 1.15% using relative standard deviation. The accuracy of the method was also verified by analyzing one standard sample before each run of experiments.

GC-MS

GC-MS was used to identify one of the transformation products detected. MSn¹ analysis was completed on each sample. Samples were analyzed using a Thermo (Trace GC 2000) gas chromatograph equipped with a RTX-5MS column (length: 30 m, film thickness: 0.25 μ m, i.d.: 0.25 mm) and a Thermo (GCQ/Polaris MS) mass spectrometer. Helium was used as carrier gas at a constant flow of 1.0 mL/min. GC injection parameters: PTV solvent split; 150°C; 150 mL/min split flow; 1.5 min splitless time. GC temperatures: 55°C isothermal 3.0 min, 10°C min to 260°C, hold 10 min, 5°C min to 275°C, hold 15 min, 0.1°C min to 275°C, hold 0.10 min. MS parameters: temperature of transfer line: 300°C, ion source: EI and CI modes, source temperature: 240°C; 3 microscans, maximum ion time of 50; scan mode: full scan; first mass: 10, last mass: 650.

Gas chromatography

GC was used to confirm the formation of volatile transformation products, such as low molecular weight alcohols. All analyses were performed on an Agilent 5890 gas chromatograph equipped with a Stabilwax column (length: 30 meters, i.d.: 0.32 mm, bonded with phase thickness of 0.25 μ m) and flame ionization (FID) detection. Helium (He) was used as carrier gas. GC conditions: 85°C oven temperature, 85°C initial temperature, 95°C injection temperature, 150°C detector temperature.

Results and Discussion

Experiments using bacteria known to remove EE2

As previously stated, strains of *R. equi* and *R. zopfii* isolated from activated sludge were shown to remove EE2 by Yoshimoto *et al.* (2004). Results for the removal of EE2 using *R. zopfii* and *R. equi* with a cosubstrate are presented in Figs. 1 and 2, respectively. Time 0 h corresponded to the addition of the EE2 solution to the bacteria during the stationary phase. Figure 1

shows *R. zopfii* removed EE2 up to 25% of its initial 0.5 mg/L concentration in 22 h. However, the removal of EE2 onto dead biomass by adsorption (adsorption control) accounted for approximately 15%. Therefore, taking into consideration a 15% adsorption removal, the actual EE2 removal percentage is approximately 10%. Conversely the removal of EE2 from an initial concentration of 1.4 mg/L was practically negligible. As observed in Fig. 2, after 65 h, *R. equi* removed EE2 up to 39% of its initial concentration of 1.4 mg/L. The adsorption control flask showed little removal demonstrating that removal mechanisms other than sorption, governed the removal of EE2.

In comparison to Yoshimoto *et al.* (2004), *R. equi* had removed 80% of the initial 100 mg/L solution of EE2 in 24 h and *R. zopfii* removed it completely within 24 h. There are several possible reasons for the higher removal of EE2 observed by Yoshimoto *et al.* The bacteria strains used by Yoshimoto *et al.* were isolated from an enrichment culture of an activated sludge from a wastewater treatment plant. Thus, the bacteria were more than likely well acclimatized to pharmaceuticals and other contaminants. Another factor to consider regarding the lower removal of EE2 obtained in the present study using *R. zopfii* and *R. equi* is the presence of a cosubstrate in comparison to the results observed by Yoshimoto *et al.* (2004) where EE2 was the only carbon source. It had been hypothesized that with a cosubstrate present in solution, efficient removal of EE2 would have been achieved through cometabolism. This, however, was not the result observed with these two strains. Most likely the micro-organisms used the readily available carbon source and EE2 was not significantly degraded by cometabolism.

Experiments using *R. rhodochrous* and *R. erythropolis*

R. rhodochrous and *R. erythropolis* were examined to determine their EE2 removal capability. *R. rhodochrous* was not particularly effective in reducing the concentration of EE2. As can be observed in Fig. 3, after 25 h, approximately 10% of

FIG. 1. Removal of 17 α -ethinylestradiol (EE2) using *R. zopfii* with adipic acid (2.5 g/L) as a cosubstrate (error bars: standard deviation based on triplicates).

FIG. 2. Removal of EE2 by *R. equi* with glucose (2.5 g/L) as a cosubstrate (error bars: standard deviation based on duplicates).

the initial concentration of 1.4 mg/L EE2 had been removed while EE2 did not undergo any removal for an initial concentration of 0.5 mg/L. No significant removal was further realized after 81.5 h. In the case of *R. rhodochrous*, it was very difficult to differentiate between adsorption and other removal mechanisms considering that a decrease in concentration of 10% was also observed in the adsorption control. The decrease in EE2 concentration can again be caused by

adsorption on the biomass rather than transformation or degradation.

Figure 4 shows the removal results of EE2 by *R. erythropolis*. For an initial EE2 concentration of 1.4 mg/L, *R. erythropolis* was capable of removing EE2 up to 35% in 9.5 h and 47% in 13 h. *R. erythropolis* also successfully removed EE2 in the 0.5 mg/L solution, eliminating 30% in 9.5 h and 48% in 13 h. Despite these promising results in a relatively short time

FIG. 3. Removal of EE2 by *R. rhodochrous* with adipic acid (2.5 g/L) as a cosubstrate (error bars: standard deviation based on triplicates).

FIG. 4. Removal of EE2 by *R. erythropolis* with adipic acid (2.5 g/L) as a cosubstrate (error bars: standard deviation based on triplicates).

period, longer exposure times to the micro-organism did not yield higher EE2 removal percentages. For both initial concentrations, approximately 47–48% of EE2 was removed in 35 h. It might be explained by the complete consumption of adipic acid, resulting in an inadequate medium composition for the micro-organism to remain healthy and continue in removing the remaining EE2. It can also be observed from Fig. 4 that sorption on biomass was not responsible for significant EE2 removal. Based on these results, among the bacteria screened *R. erythropolis*, showed the greatest capability in removing EE2; however, the removal was limited. The removal of EE2 by *R. erythropolis* was also studied in absence of cosubstrate. Results indicated a much lower removal of 10% over 75 h, indicating

that the presence of the cosubstrate played an important role in the removal of EE2 by *R. erythropolis*. These results support the hypothesis that the complete consumption of adipic acid limits further removal of EE2.

Identification of EE2 transformation products using *R. erythropolis*

Using the GC, phenol was detected in the VOC samples obtained using *R. erythropolis* after 24 and 44 h. GC-MS was used to determine the presence of EE2 transformation products in the VOC sample collected at 24 h. Blanks and matrix samples were run to apply corrections on the samples to

FIG. 5. Gas chromatography-mass spectrometry (GC-MS) chromatogram of a volatile organic compound (VOC) sample after 24 h, using *R. erythropolis* with adipic acid (2.5 g/L) as a cosubstrate.

FIG. 6. Spectrum of the peak eluted from the VOC sample after 24 h at 9.51 min.

eliminate the interference of the products associated with the bacteria growth with no exposure to EE2. Figure 5 displays the corrected GC-MS chromatogram of the sample. Prominent peaks at retention times 7.20, 9.51, and 11.69 min and others indicate the presence of new products formed in the solution containing EE2. Only the peak at 9.51 min was selected for the preliminary analysis.

Using chemical ionization, the molecular weight of the product eluting at a retention time of 9.51 min was found to be 331 amu. Figure 6 presents the spectrum of the product obtained by electron ionization at 70 eV. Figure 7 shows the structure of product determined by comparison with the NIST database and a rebuild of the molecule. Figure 7 also shows the molecular structure of EE2, indicating that the triple bond of EE2 was affected. MSn² and radiolabeled compounds would be required to further confirm the nature of this product.

Conclusions

Removal of EE2 using pure cultures in presence of an easily degraded carbon source (2.5 g/L) was first studied to

investigate the potential of some *Rhodococcus* species to degrade EE2, as well as to conduct some preliminary identification of metabolites formed, although the objective of the study was not to elucidate the biotransformation pathway of EE2.

Four bacteria were tested to investigate their potential for degrading EE2 in the presence of an easily degraded carbon source, glucose or adipic acid. *R. erythropolis* and *R. equi* demonstrated the greatest ability to remove EE2, removing up to 47 and 39% of the initial 1.4 mg/L in 13 and 65 h, respectively. Results demonstrated no significant EE2 removal using *R. rhodochrous* and *R. zopfii*. The removal of EE2 by adsorption to dead biomass was tested and in some cases, was found to be significant in its overall removal. Results obtained with *R. erythropolis* without a cosubstrate showed a reduced removal of 10% in 75 h. These results demonstrated that the presence of an easily degraded carbon source, the initial concentration of EE2 and the strains of bacteria play an important role in the removal of EE2 when exposed to micro-organisms. These results improve our understanding of the effect of variables that may influence the biotransformation of EE2 in biological wastewater treatment.

Preliminary analysis of the transformation products produced during the removal of EE2 by *R. erythropolis* was completed. GC results identified phenol as a metabolite. GC-MS analysis of the VOCs confirmed the existence of a product having a molecular weight of 331.

These results also indicate that removal of EE2 leads to high molecular weight biotransformation products rather than mineralization, and suggest that the biotransformation pathway of EE2 should be further studied to fully understand the fate of EE2 and assess the risk associated with its transformation products.

Acknowledgments

This research was supported by funding from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Eugenie Ulmer Lamothe Fund. The authors thank Mr Ranjan Roy for his analytical expertise.

Author Disclosure Statement

No competing financial interests exist.

FIG. 7. (a) Molecular structure of EE2, (b) identified EE2 metabolite with an m/z of 331.

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