The effect of ozone on the biodegradation of 17 α -ethinylestradiol and sulfamethoxazole by mixed bacterial cultures

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Abstract The potential development of antibacterial resistance and endocrine disruption has led to increased research investigating the removal of contaminants from wastewater (WW) such as sulfamethoxazole (SMX) and 17 α -ethinylestradiol (EE2). These compounds react quickly with ozone (O₃), thus ozonation during WW treatment may result in their complete removal. Also, O₃ has demonstrated the ability to increase the biodegradability of WW and certain pharmaceuticals, suggesting its potential as a pretreatment to activated sludge (AS, biological treatment). The objective of this study was to determine whether ozonation, conducted at doses lower than commonly applied to treated WW, would lead to an increased biodegradability of SMX and EE2. The results show that after ozonation performed at lab-scale the bacterial mixtures removed 5 % to 40 % more SMX; however, 2 % to 23 % less EE2 was removed, which was attributed to the observed preferential degradation of a by-product of EE2 ozonation. These results suggest that although ozonation, used as a pretreatment, was shown in literature to increase the overall biodegradability of AS as well as some specific antibiotic compounds and a blood lipid regulator, the potential for increased removal of pharmaceuticals seems to be compound-dependent and cannot yet be extrapolated to this entire class of compounds.

Keywords Ozone · Sulfamethoxazole · 17 α -ethinylestradiol · Biodegradation · Mixed bacteria

Introduction

As a result of the common use of pharmaceutical compounds worldwide, their presence in the aquatic environment has become an issue of increased interest and research. Antibiotic compounds represent one of the main concerns due to the potential development of antibacterial resistances in bacteria present in the environment and in biological wastewater (WW) treatment (activated sludge, AS) (Andersson 2003; Reinthaler et al. 2003; Volkmann et al. 2004). Estrogenic compounds represent another very important concern due to their effect on the fertility and development (endocrine disruption) of aquatic organisms at part per trillion (nanogram per liter) concentrations (Jobling et al. 2006; Vajda et al. 2008). Two of the most popularly prescribed drugs in these categories are sulfamethoxazole (SMX) and 17 α -ethinylestradiol (EE2) (Cavallucci 2007). SMX is a synthetic antibiotic that has been detected in WWs at up to microgram per liter levels and surface waters at nanogram per liter levels (Joss et al. 2005; Kolpin et al. 2002; Miège et al. 2009), while the synthetic estrogen EE2 (the main ingredient in oral contraceptive pills) has been detected at nanogram per liter levels in both surface waters and WWs (Kolpin et al. 2002; Miège et al. 2009; Ternes 1998). This is due to disposal and excretion, which has been estimated to reach from 50 % up to 90 % of the original ingested amount (Johnson and Williams 2004; Kümmerer 2009a, b) as well as incomplete removal during conventional WW treatment (Miège et al. 2009; Ternes et al. 2006).

The use of ozone (O₃) as an advanced WW treatment to increase the removal of pharmaceuticals has been the focus of numerous studies due to its ability to oxidize compounds with structures containing carbon–carbon double bonds, aromatics, hydroxyl, and amino groups (Huber et al. 2003; Ternes et al. 2006). Both SMX and EE2 possess these structural characteristics, and past studies have shown that

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they react quickly with O_3 with measured rate constants in the range of $10^5 \text{ M}^{-1} \text{ s}^{-1}$ to $10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Deborde et al. 2005; Huber et al. 2003; von Gunten 2003). Pilot and full-scale tests investigating the use of O_3 (as an advanced or tertiary treatment step) following AS during WW treatment have found that compounds with reaction rates $>10^4 \text{ M}^{-1} \text{ s}^{-1}$ can be eliminated by up to 95 % prior to final effluent discharge (Huber et al. 2005; Wert et al. 2009). While ozonation generally results in “less-active” by-products (Ternes et al. 2003), it has been shown that the reaction of SMX with O_3 can result in the formation of another antibiotic (sulfanilamide) (Yargeau et al. 2008), and the ozonation of EE2, which reduces estrogenicity, has been recently shown to increase toxicity with respect to testosterone secretion by fetal rats (Larcher et al. 2012). Also, it has been shown, using indicators such as biological oxygen demand (BOD) and chemical oxygen demand (COD), that treatment with highly concentrated ozone increases the biodegradability of WW (Beltrán et al. 1999; Ternes et al. 2006) as well as specific compounds such as penicillin, enrofloxacin, ceftriaxone, and bezafibrate (Alaton et al. 2004; Balcioglu and Otker 2003; Dantas et al. 2007, 2008). This has led to the hypothesis that the application of small O_3 doses prior to biological WW treatment may be implemented to lower estrogenic and antibacterial activity before exposure to AS microbial populations and increase biodegradation and removal of pharmaceutical compounds and/or potentially harmful ozonation by-products formed (Alvares et al. 2001; Dodd et al. 2009; Zhang et al. 2008).

The aim of this study was to investigate the effect of a low dose of O_3 applied prior to biological treatment on the biodegradation of SMX and EE2 at lab-scale using controlled mixtures of pure bacterial cultures that have been previously detected in AS and pharmaceutical WW effluent (Rani et al. 2008; Seviour et al. 2008). Although these pure bacterial mixtures are not representative of the whole range of microorganisms present in actual WW treatment systems, this experimental approach is more realistic than using single-strain pure culture studies and was required in order to maintain a controlled and reproducible system that cannot be obtained using inoculum from AS treatment. This approach allows the study of multiple strains at the same time while maintaining the possibility of comparing results obtained under different experimental conditions.

Materials and methods

Chemical reactants and reagents

All chemicals used were of reagent or HPLC grade. EE2 (CAS 57-63-6, purity 98 %), SMX (CAS 723-46-46), ammonium nitrate (NH_4NO_3), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$),

magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), ammonium acetate, formic acid, and glucose assay kit (GAHK-20) were purchased from Sigma-Aldrich, Canada. Iron sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), ethylene diamine tetra-acetic acid (EDTA), sodium hydrogen monophosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), yeast extract, and acetonitrile (ACN) were purchased from Fisher Scientific, Canada. Anhydrous ethanol (EtOH) was purchased from Commercial Alcohols, Canada. McFarland Standard #2 was obtained from bioMérieux, France; BHI (Brain Heart Infusion) Agar was obtained from Becton-Dickinson & Co., Canada; and glucose was obtained from A & C American Chemicals Ltd., Canada.

Stock solutions of SMX and EE2 were prepared in reverse osmosis (RO) water at the following concentrations: 60 mg/L SMX (6 % EtOH) and 50 mg/L EE2 (5 % EtOH), respectively. A stock solution of SMX (60 mg/L) without EtOH was also prepared in order to determine the effect of EtOH on biodegradation. Stock ozone solutions were prepared as described in “Batch ozonation”.

Batch ozonation

Stock O_3 solutions were made by sparging O_3 gas produced by an Ozomax generator and a pure oxygen gas feed through a 500-mL gas washing bottle filled with RO water and surrounded by ice. The dissolved O_3 was determined spectrophotometrically by measuring the absorbance at 258 nm ($\epsilon = 2,950 \text{ M}^{-1} \text{ cm}^{-1}$) (Bader and Hoigné 1981). Due to the nature of batch ozonation and the desired concentrations for the ozonated stock SMX and EE2 solutions, it was necessary to use aliquots of highly concentrated SMX or EE2 solutions made in ethanol (1,000 mg/L). The volume of O_3 stock solution required to achieve a 5 mg/L O_3 dose was added to the experimental SMX (60 mg/L) or EE2 (50 mg/L) stock solutions (containing 6 % and 5 % EtOH, respectively) and mixed vigorously until all the O_3 had reacted with SMX or EE2 and had dissipated from the solution (approximately 2 h). These ozonated SMX and EE2 solutions were then stored at 4 °C until needed for the biodegradation experiments. Based on the results of a previous study (Rodayan et al. 2010), it was assumed that the reaction pathways between O_3 and SMX or EE2 at these higher concentrations were identical to those that would take place at lower concentrations such as those encountered in municipal WW. The addition of small aliquots (10 % of the total working volume) of these concentrated ozonated stock SMX or EE2 solutions to the experimental flasks resulted in the desired conditions of 6 mg/L SMX or 5 mg/L EE2 treated with 0.5 mg/L O_3 . This ten-time dilution of the ozonated stock solutions ensured that SMX, EE2, and EtOH (0.6 % and 0.5 % v/v) were at levels that were not observed to be growth-limiting. It should also be noted that the reaction rate of EtOH with O_3 ranges from 0.45 to

$0.77 \text{ M}^{-1}\text{s}^{-1}$ (Yao and Haag 1991), which is much lower than that of SMX ($2.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) (Huber et al. 2003) or EE2 (1.83×10^5 to $3.65 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) (Deborde et al. 2005). It is thus expected that EtOH would not impede the reaction of either compound with O_3 . This hypothesis was confirmed by gas chromatography analysis indicating no changes in the concentration of EtOH after ozonation.

Biological and chemical oxygen demand (BOD/COD)

The 5-day biological oxygen demand (BOD_5) was measured according to Standard Method 5210D (Standard Methods for the Examination of Water and Wastewater) using Polyseed™ BOD seed inoculum. The dissolved oxygen was measured using a BOD self-stirring probe (Thermo Scientific, 13-620-SSP) and an Accumet Excel XL60 Dual Channel pH/Ion/Conductivity/DO meter (Fisher Scientific). COD was measured according to Standard Method 5220D using a HACH Digital Reactor Block 200 (DRB 200) and a HACH spectrophotometer DR/2500. The BOD_5 :COD ratio was used as a tool to evaluate biodegradability.

Individual bacteria

The pure bacterial cultures used in this study were obtained from Cedarlane® Canada and stored at -80°C in a BHI/glycerol mixture. Each bacteria was thawed and grown individually in BHI broth for 24 h at 26°C and 150 rpm (INNOVA® 44 Incubator Shaker Series) and then plated and grown on BHI agar. After incubation for 24 to 48 h at 26°C in the dark, the agar plates were stored at 4°C . The seven bacteria used in this study were (ATCC#): *Bacillus subtilis* (6051), *Pseudomonas aeruginosa* (PA01), *Pseudomonas putida* (12633), *Rhodococcus equi* (13557), *Rhodococcus erythropolis* (4277), *Rhodococcus rhodochrous* (13808), and *Rhodococcus zopfii* (51349). These bacteria were chosen due to their previous detection and isolation from WWTP-AS and for their facility treating pharmaceutical effluent and/or their demonstrated ability to degrade EE2 or SMX (Gauthier et al. 2010; O'Grady et al. 2009; Rani et al. 2008; Seviour et al. 2008; Yoshimoto et al. 2004).

Mixed bacterial groups and inhibition

Prior to mixing the bacteria together, inhibition tests were conducted as described elsewhere (Larcher and Yargeau 2011) in order to determine the final bacterial mixtures used in this study. Based on the results, two different bacterial mixtures were studied in order to consider all the strains while avoiding inhibition problems. Group 1 consisted of *P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis*, and *R. rhodochrous*; and Group 2 consisted of *B. subtilis*, *P. putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, and *R. zopfii*.

Biodegradation

Experiments were carried out in duplicate using the bacterial mixtures in Group 1 and Group 2 and SMX or EE2 solutions with and without pre-treatment with O_3 , and with or without an additional source of easily degraded carbon (glucose). For each bacteria, the growth on BHI agar plates was used to make cellular standards (McFarland Standard #2; 3×10^8 cells/mL) in 6 mL of a 0.85 % sterile sodium chloride solution. These were used to pre-inoculate 250-mL Erlenmeyer flasks (working volume of 100 mL) containing minimum mineral salt media (MMSM), 6 mg/L SMX, or 5 mg/L EE2 (from the addition of a small aliquot of 60 mg/L SMX or 50 mg/L EE2 stock solutions with and without prior O_3 treatment) and were placed in a dark incubator shaker (24 h, 26°C , 150 rpm). This pre-inoculant was prepared in order to acclimate each of the seven bacteria to the test conditions. The MMSM used was composed of $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (0.018 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.013 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.013 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 g/L), Na_2HPO_4 (7.5 g/L), KH_2PO_4 (5 g/L), and NH_4NO_3 (5 g/L). Yeast extract was also added to the aqueous matrix at 0.6 g/L of the working volume (Gauthier et al. 2010). Equal volumes of each pre-inoculant individual bacterial growth were used to inoculate the 500-mL experimental Erlenmeyer flasks (working volume of 350 mL). The total volume of the mixed bacterial pre-inoculants added to each experimental flask was 70 mL (20 % of the total working volume). The experimental flasks contained the same aqueous matrix as the pre-inoculant flasks. It should be noted that the aliquots of SMX or EE2 stock solutions added to each experimental flask were based on achieving initial concentrations of 6 mg/L SMX or 5 mg/L EE2 in a total working volume of 350 mL assuming that no residual SMX or EE2 remained in the transferred inoculum from the pre-inoculant flasks. However, SMX and EE2 were consistently transferred from the preinoculant to the experimental flasks, explaining the observed initial concentrations that were slightly higher than 6 mg/L SMX or 5 mg/L EE2. For ease of analysis, the SMX and EE2 concentrations used in this study are higher than those encountered in WW, which has also occurred in previous studies where SMX concentrations ranged from 3.76 mg/L to 100 mg/L (Alexy et al. 2004; Drillia et al. 2005; Gauthier et al. 2010) and EE2 concentrations ranged from 3.5 to 100 mg/L (Weber et al. 2005; Yoshimoto et al. 2004).

Control experiments were carried out with each mixed bacteria group to verify that any metabolites detected were due solely to SMX or EE2 transformation by conducting the above described biodegradation experiments with no SMX or EE2. Adsorption onto the bacteria was also found to be within the error of analysis ($\pm 2\%$) as indicated by experiments conducted with bacteria killed via autoclaving

immediately after pre-inoculation. Growth tests were carried out on BHI agar plates at the SMX, EE2, and EtOH concentrations used in this study; no inhibitory effect on the growth of the seven bacteria was observed.

Analytical methods

Bacterial growth was measured via the optical density at 540 nm ($O.D_{540}$) using a Thermo Evolution 300 UV–Visible Spectrophotometer. To monitor the concentration of SMX or EE2, 2 mL aliquots were removed from each experimental flask over time and centrifuged at 10,000 rpm for 10 min (Thermo IEC MicroCL 21). The supernatant was syringe-filtered with a 0.22- μ m PVDF filter directly into an amber vial for HPLC analysis.

Determination of SMX was carried out using an Agilent 1200 HPLC equipped with a Diode Array Detector (DAD) at a wavelength of 273 nm and a XDB Phenyl column (150 $\times$ 4.6 mm, 3.5 μ m). Mobile phases consisted of 20 mM NaH_2PO_4 (pH 2.8 using phosphoric acid) and ACN using a gradient from 30 % to 45 % ACN over 10 min; the method used a column temperature of 40 °C, an injection volume of 10 μ L, and a flow rate of 0.7 mL/min. The limit of detection and limit of quantitation were 2 μ g/L and 7 μ g/L, respectively.

Determination of EE2 was carried out using the same HPLC and detector at a wavelength of 279 nm and a Zorbax Eclipse Plus C18 column (150 $\times$ 4.6 mm, 3.5 μ m). Mobile phases consisted of 0.5 mM ammonium acetate (pH 2.8 using formic acid) and ACN using a gradient from 30 % to 60 % ACN over 20 min; the method used a column temperature of 28 °C, an injection volume of 50 μ L, and a flow rate of 0.8 mL/min. The limit of detection and limit of quantitation were 125 μ g/L and 417 μ g/L, respectively.

Determination of ethanol in the EE2 and SMX stock solutions before and after ozonation was carried out using an HP 5890 Gas Chromatograph equipped with a flame ionization detector (FID) and a Restek Stabilwax® column (30 m $\times$ 0.32 mm, 0.25 μ m). The carrier gas was helium at a constant flow of 30 mL/min, with an injection volume of 2 μ L (direct injection). The detector and injector temperature was 175 °C with an oven temperature of 130 °C.

Results

Effect of EtOH on SMX biodegradation

Due to the low solubility of EE2 in pure RO water, it was not possible to carry out experiments without diluting a concentrated stock solution made in EtOH. However, it was possible to make two different 60 mg/L SMX solutions—one in pure RO water and another made by diluting a

1,000 mg/L SMX solution (made in 100 % EtOH)—which were each used in separate biodegradation experiments to compare the effect of EtOH on bacterial growth and SMX removal. Aliquots of the SMX stock solution were added to each pre-inoculant and experimental flask in order to achieve 6 mg/L SMX and 0.6 % (v/v) EtOH for the flask prepared using the stock solution containing the solvent. This amount of EtOH was not inhibitory; however, its presence had a significant effect on both the bacterial growth and SMX removal. Without EtOH the maximum SMX removal achieved by either mixed group of bacteria after 300 h was 5 %; however, with EtOH, Group 1 achieved approximately 40 % SMX removal and Group 2 achieved 15 % SMX removal. There was also a metabolite peak eluted 0.3 min before SMX during HPLC analysis in both groups grown in the presence of EtOH, which was four times larger in Group 1. Without EtOH, both Group 1 and Group 2 grew to a maximum $O.D_{540}$ of 0.6, while the maximum $O.D_{540}$ achieved in the presence of EtOH by both groups was approximately 2.5. These results clearly show that the presence of EtOH enhanced the growth of both mixed bacterial groups (more than four times) and increased SMX removal by a factor of 3 (Group 2) to 8 (Group 1).

Effect of O_3 on the BOD_5 :COD

The change in biodegradability of a 6 mg/L SMX solution in pure RO water (with no additional carbon sources, i.e., no EtOH) due to the application of 0.5 mg/L O_3 was estimated using the measured BOD_5 :COD. This analysis was carried out in triplicate with results showing that the BOD_5 :COD increased from 0.2 ± 0.1 without O_3 to 5.1 ± 0.4 after O_3 treatment. The BOD_5 :COD was not measured for EE2 because it was necessary to dilute a concentrated EE2 solution made in EtOH to achieve the desired 5 mg/L concentration. This would result in the presence of 0.5 % (v/v) EtOH, which would render the BOD_5 and COD analyses ineffective.

Effect of O_3 on SMX biodegradation

Without O_3 pre-treatment, the growth of each mixed bacterial group was virtually identical, attaining a maximum $O.D_{540}$ of approximately 2.5 (Group 2) to 2.7 (Group 1). However, these similar growth patterns did not result in the same observed SMX removals (Fig. 1a and b). Group 1 achieved approximately 42 % SMX removal after 300 h, and a metabolite peak was detected during HPLC analysis, which eluted 0.3 min earlier than SMX (Fig. 1a). Group 2 achieved less SMX removal after 300 h (15 %) with a metabolite peak detected at the same time as Group 1, but approximately five times smaller (Fig. 1b).

The results show that after ozonation (where 10 % of the initial SMX disappeared due to reaction with O_3), the growth of each bacterial mixture was relatively unchanged, with maximum $O.D_{540}$ values ranging from 2.4 to 2.7. The SMX removed via biodegradation after 300 h by Group 1 was increased from 42 % to 47 % after ozonation (Fig. 1a). When combined with the 10 % SMX removal due to reaction with ozone, the overall SMX removal achieved by Group 1 was 57 %, 15 % greater than without O_3 pre-treatment. The results of Group 2 were more impressive with the SMX removal increasing from 15 % to 55 % after ozonation (Fig. 1b). When added to the removal resulting from reaction with O_3 , the overall SMX removal achieved by Group 2 was 65 %. For both Group 1 and Group 2, the same metabolite peak was detected as without O_3 pre-treatment.

Effect of O_3 on EE2 biodegradation

Without O_3 pretreatment, the growth of the bacteria reached maximum $O.D_{540}$ values of 2.2 (Group 2) to 2.7 (Group 1), and each bacterial mixture removed the same amount of EE2 after 300 h (42–43 %).

After ozonation, 45 % of the initial EE2 disappeared and an EE2+ O_3 by-product peak was consistently detected at the wavelength of HPLC analysis. The growth of the bacteria was relatively unchanged after ozone treatment with maximum $O.D_{540}$ values of 2.6 (Group 1) and 2.2 (Group 2). The EE2 removal achieved by both mixed bacterial groups did not increase after O_3 treatment; Group 1 achieved 20 % EE2 removal after 300 h (compared to 43 % without O_3) (Fig. 2a), while Group 2 removed 40 % EE2 (compared to 42 % without O_3) (Fig. 2b). However, the overall EE2 removals achieved were greater than those without O_3 pre-treatment, since almost half of the initial EE2 disappeared after reaction with O_3 .

Effect of an additional carbon source on SMX and EE2 biodegradation

Experiments were carried out with an additional readily degradable carbon source (0.5 g/L glucose) to investigate the effect on bacterial growth and removal of SMX and EE2 (both with and without ozone pre-treatment). For all of the conditions investigated, the addition of glucose did not greatly increase the bacterial cell density (not shown) which indicates that it did not promote a detectable increase in bacterial growth. Despite this similar bacterial growth of the mixed bacteria, there were some differences observed in the SMX and EE2 removals (Table 1). Without ozone pre-treatment, Group 1 had similar SMX removal regardless of glucose (40–42 %) but drastically less EE2 removal with glucose addition (25 % vs. 43 % without glucose). The EE2 removed by Group 2 was not affected by glucose addition

(42–43 % removal) but there was two times more SMX removed with glucose present (31 % vs. 15 % without glucose).

After ozonation of the SMX and EE2 solutions, the observed effect of glucose addition was different (Table 1). While Group 1 achieved similar removals of EE2 (20–17 %) and only slightly higher SMX removal with glucose (59 % vs. 47 % without glucose), Group 2 removed significantly less EE2 and SMX in the presence of glucose (20 % each vs. 40 % EE2 removal and 55 % SMX removal without glucose).

Glucose concentrations were measured at three different times throughout the experiments (0, 120, and 300 h) and the results (not shown) illustrate that the overall glucose consumption by Groups 1 and 2 was identical regardless of ozonation for both SMX (98 ± 0.4 % glucose consumption) and EE2 (98.5 ± 0.3 % glucose consumption).

Discussion

SMX degradation

The presence of 0.5 % (v/v) EtOH was observed to result in enhanced bacterial growth and increased SMX removal; this is not unexpected since a previous study observed the same trends due to the presence of methanol (Radke et al. 2009). Therefore, it should be kept in mind that the observed removals of SMX during the biodegradation experiments in this study may be elevated due to the presence of EtOH. This is not an unrealistic set of conditions as this phenomenon might be observed in treatment plants treating WW containing a variety of additional sources of carbon.

The measured SMX percent removals (15 % to 42 %) are lower than previously published results of lab-scale experiments conducted with various AS samples (Drillia et al. 2005; Perez et al. 2005) where up to complete SMX removal was observed. However, they are closer to the SMX removal predicted by the biological degradation constant (k_{biol}) determined at batch-scale using AS samples ($0.1 < k_{biol} < 1 \text{ Lg}_{ss}^{-1} \text{ day}^{-1}$), which suggested approximately 20 % removal would occur via biodegradation (Joss et al. 2006). The difference between the SMX degradation achieved by the two mixed groups may be a result of the different microbial populations, specifically the presence (Group 1) or absence (Group 2) of *P. aeruginosa* which is known to produce the enzyme arylamine N-acetyltransferase (BRENDA: The Comprehensive Enzyme Information System). Based on the specificity of this enzyme to attack SMX, the mixture containing *P. aeruginosa* (Group 1) would be expected to be more successful at degrading SMX than the one without *P. aeruginosa* (Group 2), which was observed in this study.

Prior to carrying out biodegradation experiments to investigate the impact of ozone pre-treatment, an initial

Fig. 1 Sulfamethoxazole (SMX) removal by mixed bacterial cultures with and without ozone (O_3) pre-treatment. **a** Group 1 (*P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis*, and *R. rhodochrous*); **b** Group 2 (*B. subtilis*, *P. putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, and *R. zopfii*)

analysis was performed using the measured $BOD_5:COD$ of a 6 mg/L SMX solution with and without ozone treatment. In past studies, this ratio has been shown to increase after ozonation of WWs (Beltrán et al. 1999) and SMX solutions (Dantas et al. 2008), which predicts increased biodegradability. In this study, the average $BOD_5:COD$ (performed in triplicate) of the SMX solutions increased by a factor of 25 after reaction with O_3 , which indicates that the biodegradability of the solution was greatly increased.

It was expected, due to the increased $BOD_5:COD$ measured after O_3 application, that ozonation would result in the observed increase in biodegradation of SMX. This increased biological removal of SMX observed after ozonation may have been due to the formation of SMX+ O_3 by-products (undetected by the analytical method used) that encouraged cometabolism of SMX. Several potential ozone by-products have been detected (Dantas et al. 2008) and identified (Rodayan et al. 2010) under different conditions in other studies, and it is possible that some of these previously

identified by-products of SMX ozonation were formed in this study. It has also been shown that ozonation of SMX results in decreased antibacterial activity (Dodd et al. 2009) and the formation of bioactive by-products that do not negatively affect the proliferation of mammalian cells and may even stimulate cell growth (Yargeau et al. 2008). These post-ozonation properties may have added to the observed increase in SMX removal by the bacterial mixtures after reaction with O_3 .

The potential structures of the compounds contained in the detected metabolite peak formed during the biodegradation of SMX by Groups 1 and 2 were proposed in a previous study based on the enzymes produced by *Rhodococcus* species in general and *R. equi* specifically (Larcher and Yargeau 2011). The enzyme arylamine N-acetyltransferase is produced by *Rhodococcus* species; this enzyme specifically attacks compounds containing aromatic amine structures and is known to use SMX as a substrate (BRENDA: The Comprehensive Enzyme Information System). Since both mixed groups contain three to four different types of

Fig. 2 17 α -ethinylestradiol (EE2) removal by mixed bacterial cultures with and without ozone (O_3) pre-treatment. **a** Group 1 (*P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis*, and *R. rhodochrous*); **b** Group 2 (*B. subtilis*, *P. putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, and *R. zopfii*)

Table 1 Summary of percent removals of EE2 and SMX after 300 h by mixed bacterial cultures in Group 1 (*P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis*, and *R. rhodochrous*) and Group 2 (*B. subtilis*, *P.**putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, and *R. zopfii*) with and without ozone (O₃) pre-treatment and an additional source of readily degradable carbon (0.5 g/L glucose)

Mixed bacteria	Percent removal after 300 h ^a			
	EE2		SMX	
	No glucose	With glucose	No glucose	With glucose
Group 1	43 %±3.5 %	25 %±1.7 %	42 %±0.3 %	40 %±3 %
Group 2	42 %±1.5 %	43 %±1.7 %	15 %±0.2 %	31 %±0.4 %
Group 1 (pre-O ₃)	20 %±1.5 %	17 %±0.8 %	47 %±1.2 %	59 %±0.1 %
Group 2(pre-O ₃)	40 %±1.5 %	20 %±1.2 %	55 %±0.2 %	20 %±1.5 %

^a Average of duplicate experiments ± range in values

Rhodococcus bacteria, it is hypothesized that the production of this enzyme would result in a metabolite with an acetyl group attached to the aromatic amine in SMX as was proposed previously. This same study also suggested that *R. equi* is capable of producing several different enzymes that might lead to the hydrolysis of the proposed acetylated metabolite to potentially form an alcohol derivative. Both of these potential metabolite structures are more polar than SMX, which would result in their observed earlier elution during the HPLC analysis used.

EE2 degradation

The approximately 40 % EE2 removal via biodegradation that was observed in this study using the two defined bacterial mixtures (Groups 1 and 2) is not completely surprising as a previous lab-scale investigation using AS samples predicted that EE2 would be partially removed during biodegradation (Joss et al. 2006). However, despite this prediction of partial biodegradation, there have been variable results reported from batch experiments using AS ranging from 0 (Weber et al. 2005) to 100 % (Hashimoto and Murakami 2009) EE2 removal. There are also conflicting results from past studies investigating the role played by the type of AS on EE2 degradation. While some studies have shown nitrifying AS to degrade EE2 more successfully than the heterotrophic bacteria in conventional AS (Ren et al. 2007; Vader et al. 2000), others have shown heterotrophs to be more effective at degrading EE2 (Gaulke et al. 2008) and its metabolites (Shi et al. 2004). Thus, past results are highly variable (due to differences in experimental protocols and bacterial population in AS samples), but this study demonstrates that EE2 is partially biodegraded by the controlled mixtures of heterotrophic bacteria in Group 1 and Group 2. This may be due to the presence of up to four *Rhodococcus* species in each mixture, since this genus is capable of degrading organic compounds that are structurally similar to EE2 (Larkin et al. 2005; Martinková et al. 2009). Also, *R. equi*

and *R. zopfii* have specifically demonstrated EE2-degrading capabilities in past studies (Yoshimoto et al. 2004; O'Grady et al. 2009).

The decreased biodegradation of EE2 after ozonation may be due to the formation of the EE2 ozonation transformation product that was detected as a peak that was eluted 5 min prior to EE2 during HPLC analysis. Figure 2a and b show that this transformation product was degraded quickly by both bacterial groups suggesting that it was preferentially degraded by the bacteria over EE2. HPLC analysis conducted at a different wavelength of detection (250 nm) showed that another EE2 ozonation transformation product was formed (O₃ by-product B) which grew in size as the other one was rapidly degraded (O₃ by-product A). Another metabolite having a different HPLC retention time that formed after 24 h in Group 1 was also detected. The transformation product (O₃ by-product B) and metabolite detected at a wavelength of 250 nm persisted throughout the biodegradation experiment but appears to have been slightly degraded (Fig. 3a and b). The formation of a persistent metabolite in Group 1 only may be the cause of the significantly reduced EE2 removal after O₃ treatment in this bacterial mixture compared to Group 2. Thus, the decreased EE2 biodegradation observed after ozone treatment may have been due to the preferential quick degradation of the first transformation product (O₃ by-product A) combined with the slow degradation of the other transformation product (O₃ by-product B) and metabolite. The HPLC retention time of these two transformation products corresponds to those observed in a previous study using the same analytical method in which the degradation of EE2 due to ozonation and the resulting impacts on estrogenicity and toxicity were investigated (Larcher et al. 2012). Results showed that these peaks were comprised of several compounds of which only two were successfully identified. These transformation products were both muconic acid derivatives with open phenolic ring structures and molecular weights of 302 and 344 g mol⁻¹. The results of another study modeling the effect

Fig. 3 Formation and removal of ozone by-products of 17 α -ethinylestradiol (EE2) and a metabolite by mixed bacterial cultures: **a** Group 1 (*P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis* and *R. rhodochrous*) and **b** Group 2 (*B. subtilis*, *P. putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, and *R. zopfii*). HPLC analysis conducted at a detection wavelength of 250 nm

of compound structure on biodegradability showed that closed aromatic rings tended to retard the predicted biodegradation (Loonen et al. 1999); this may be one of the reasons for the observed preferential biodegradation of the transformation products compared to EE2.

Impact of glucose addition

This study demonstrates that the presence of a source of readily degradable carbon (in addition to the pharmaceutical compound being studied and the ethanol and yeast present in the aqueous matrix) does not always result in a predictable trend in the biodegradation of pharmaceutical compounds. In some cases the overall removal may increase, while in others the opposite may occur and there can also be no observed

change. The effect of glucose addition seems to depend on the type of compound being degraded and the composition of the mixed bacterial culture, which is known to be highly variable from one AS treatment plant to another.

It is possible that, depending on the bacterial species present in the mixed culture and the pharmaceutical compound being degraded, the addition of a readily degradable carbon source and its subsequent consumption may lead to increased removal of the pharmaceutical compound via cometabolism or decreased removal due to preferential consumption of the readily degradable carbon source or a transformation product. Exploration of these possibilities was not within the scope of this study but should be considered for future work.

Our results agree with the variability observed in previously reported batch studies carried out with AS samples. In these

studies, SMX removals were both enhanced (Alexy et al. 2004) and inhibited (Drillia et al. 2005) by additional sources of carbon and 20 times less EE2 removal was observed in the presence of excess carbon (Muller et al. 2010). This also illustrates that variation in the microbial population of a mixed consortium of bacteria, whether AS or pure cultures, can cause drastic differences in the biodegradation trends observed. This suggests that the bacterial species making up the mixture being used must be identical in order to compare the results of lab-scale degradation studies.

Overall implications

This study demonstrates that the application of ozone (O_3) may result in increased biodegradation of pharmaceutical compounds; however, the results vary depending on the microbial population, the presence of additional carbon sources, and the nature of the pharmaceuticals. The biological removal of the antibiotic SMX generally increased after ozonation, but the mechanisms causing this observation require further study. For the estrogen EE2, where the reaction with O_3 results in the formation of a transformation product that was preferentially degraded by the bacteria being studied, O_3 pre-treatment resulted in decreased biodegradation of EE2 (the parent compound). It might be possible that a larger O_3 dose would result in the complete disappearance of EE2 and the formation of more biodegradable transformation products, which would, in turn, be quickly degraded by the bacteria. These results imply that the use of ozone as a pre-treatment to biological treatment (at doses less than or equal to those used for tertiary treatment following AS) can potentially be considered as an alternative way to implement O_3 and achieve added benefits during WW treatment. In addition, the fact that opposing results were obtained for EE2 and SMX indicate that further studies are required to assess the potential of ozonation prior to biological treatment for this important class of contaminants of emerging concern.

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