Biodegradation of 17α-ethinylestradiol by heterotrophic bacteria

Simone Larcher and Viviane Yargeau*

Department of Chemical Engineering, McGill University, 3610 University Street, Montréal, Québec, Canada, H3A 2B2

*viviane.yargeau@mcgill.ca, +1-514-398-2273 (tel), +1-514-398-6678 (fax)

ABSTRACT

The presence of the synthetic estrogen 17α-ethinylestradiol (EE2) in the environment is of increasing concern due to the endocrine disruption of aquatic organisms. Incomplete removal from wastewater (WW) is one of the main sources of EE2 in aquatic ecosystems, thus improving processes like biological WW treatment / activated sludge (AS) is becoming significantly important. There are opposing results regarding EE2 biodegradability by AS; one discrepancy is the efficacy of heterotrophic bacteria. This research demonstrated the ability of heterotrophs commonly present in AS (*B. subtilis*, *P. aeruginosa*, *P. putida*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, *R. zopfii*) to remove EE2. *R. rhodochrous* was the most successful with no detectable EE2 after 48 hours; the other bacteria achieved 21% to 61% EE2 removal. No additive or synergistic effects were observed due to the combination of the bacterial cultures with maximum EE2 removals of 43% after 300 hours.

Capsule: *Heterotrophic bacteria, especially Rhodococcus species, are capable of successfully degrading* 17α-ethinylestradiol (EE2).

Keywords: 17α-ethinylestradiol (EE2), estrogens, biodegradation, heterotrophic bacteria, *Rhodococcus*

Introduction

The presence of pharmaceutical compounds in the aquatic environment has become an increasingly important issue due to their extensive use and disposal worldwide. Estrogenic compounds represent one of the largest concerns due to their effect on the fertility and development (endocrine disruption) of aquatic organisms at part-per-trillion (ng/L) levels (Daughton, 2003; Jobling et al., 2006; Ternes, 2006; Vajda et al., 2008). The synthetic estrogen 17α-ethinylestradiol (EE2), the main ingredient of commonly used oral contraceptive pills, has been detected in wastewaters and surface waters at ng/L levels (Cicek et al., 2007; Gomes et al., 2003; Joss et al., 2005; Kolpin et al., 2002; Lai et al., 2002; Miège et al., 2009; Petrovic et al., 2002; Ternes, 1998; Ternes et al., 1999a; Ternes et al., 1999b) due to disposal and excretion, which can reach up to 50% of the ingested dose (Hannah et al., 2009; Johnson and Williams, 2004). The presence of EE2 in the aquatic environment is worrisome due to its classification as a toxic compound to aquatic organisms that may cause long-term (chronic) effects (Carlsson et al., 2006) such as endocrine disruption and reproductive disorders (Aravindakshan et al., 2004; Cevasco et al., 2008; Lange et al., 2009; Vos et al., 2000). In addition, EE2 exposure to male rats has been shown to permanently disrupt the reproductive tract (Howdeshell et al., 2008) as well as significantly lower testosterone secretion during fetal development (Larcher et al., 2012).

EE2 removal rates in wastewater treatment plants (WWTPs) have been investigated and measured to range from 34% to 98% (Baronti et al., 2000; Cicek et al., 2007; Clouzot et al., 2008; Johnson and Sumpter, 2001; Lai, 2000; Miège et al., 2009), thus there is incomplete and inconsistent EE2 removal resulting in measured WWTP effluent concentrations of up to 62 ng/L (Lai et al., 2002). This has led to estrogenic WWTP effluents (Pauwels et al., 2008) and is estimated to contribute up to 50% of the overall estrogenicity in surface waters (Clouzot et al., 2008), resulting in observations of vitellogenin (egg yolk precursor protein expressed in females)

production in male fish exposed to WWTP effluent (Martinović et al., 2007) and the presence of significantly less male fish downstream of WWTP effluent discharge sites (Vajda et al., 2008).

This incomplete removal during wastewater treatment has resulted in research focused on optimizing the removal of estrogens like EE2; one of the primary areas of study has been biological degradation and the importance of different types of bacteria present in activated sludge (AS), especially the roles played by heterotrophic and nitrifying bacteria (Silva et al., 2012). Lab-scale studies have had inconsistent results regarding the biological degradation of EE2 by conventional AS samples, varying from complete removal (Hashimoto and Murakami, 2009) to none at all (Weber et al., 2005). There are also mixed results regarding the EE2-degrading capabilities of heterotrophic versus nitrifying bacteria (Clouzot et al., 2010; De Gusseme et al., 2009; Gaulke et al., 2008; Ren et al., 2007; Shi et al., 2004; Skotnicka-Pitak et al., 2009; Vader et al., 2000; Yi and Harper, 2007) as well as those of specific *Rhodococcus* species (O'Grady et al., 2009; Yoshimoto et al., 2004).

The aim of this research was to evaluate the potential of pure strains of heterotrophic bacteria (commonly present in AS) to degrade EE2 and to determine if this biodegradation potential is changed when these pure cultures are combined to form mixtures.

Materials and Methods

Chemicals

All chemicals used were reagent or HPLC grade. The EE2 (CAS 57-63-6, purity 98%), ammonium nitrate (NH_4NO_3), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and formic acid were purchased from Sigma-Aldrich, Canada. The iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ethylene diamine tetra-acetic acid (EDTA), sodium hydrogen monophosphate (Na_2HPO_4), sodium dihydrogen phoshate (NaH_2PO_4), yeast extract, and acetonitrile (ACN) were purchased from Fisher Scientific, Canada. Anhydrous ethyl alcohol was purchased from Commercial Alcohols, Canada. The McFarland Standard #2 was obtained

from Biomérieux, France and BHI (Brain Heart Infusion) Agar was obtained from Becton-Dickinson & Co., Canada.

Bacteria and cultivation

The pure heterotrophic 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 hours at 26°C and 150 rpm (INNOVA® 44 Incubator Shaker Series) and then plated on BHI agar. After incubation for 24 to 48 hours at 26°C in the dark, the agar plates containing each bacterial growth were stored in the fridge 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 municipal WWTP AS or an industrial WW facility treating pharmaceutical effluent, and/or their demonstrated ability to degrade EE2 in the case of *P. aeruginosa*, *R. equi*, *R. erythropolis*, and *R. zopfii* (O'Grady et al., 2009; Pauwels et al., 2008; Rani et al., 2008; Seviour et al., 2008; Yoshimoto et al., 2004).

Inhibition tests and mixed bacterial cultures

In a previous study, inhibition tests were conducted to determine the compatibility of the seven bacteria considered here to grow together (Larcher and Yargeau, 2011). This ensured that the results observed with mixed cultures would be due to the combined activities, and not impacted by growth inhibition, of the different bacterial species. The two bacterial mixtures used in the current study are based on these previously published results and the following bacterial mixtures were used: 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

Biodegradation experiments were carried out in duplicate using each of the seven bacteria individually, followed by the two bacterial mixtures (Group 1 and Group 2) outlined in the previous section. The bacterial growth on BHI agar was used to make cellular standards of each individual bacteria (McFarland Standard #2; $3x10^8$ cells/mL) in 6 mL of 0.85% sterile saline solution. These cellular standards were then used to pre-inoculate 250 mL Erlenmeyer flasks (working volume of 100 mL) containing minimum mineral salt media (MMSM) and 5 mg/L EE2 and were placed in a dark incubator shaker (24 hours, 26°C, 150 rpm). This pre-inoculant was prepared in order to acclimate each of the 7 bacteria to the test conditions. The MMSM used was composed of $Na_2EDTA{\cdot}2H_2O$ (0.018 g/L), $FeSO_4{\cdot}7H_2O$ (0.013 g/L), $CaCl_2{\cdot}2H_2O$ (0.013 g/L), $MgSO_4{\cdot}7H_2O$ (0.25 g/L), Na_2HPO_4 (7.5 g/L), KH_2PO_4 (5 g/L), NH_4NO_3 (5 g/L), to which yeast extract (0.6 g per liter of the working volume) was added. For ease of analysis and experimentation, the initial EE2 concentration used in this study is approximately 3 orders of magnitude greater than levels observed in wastewater and surface water. This has also occurred in other studies where the EE2 concentrations studied were higher in order to quantify biodegradation and adsorption during biological removal of EE2 and ranged from 3.5 to 100 mg/L (Clouzot et al., 2010; Weber et al., 2005; Yoshimoto et al., 2004). While the selected concentration of EE2 does not represent that observed in the aquatic environment or WWTPs, the results of this study provide useful information regarding the EE2 degrading capabilities of heterotrophic bacteria individually and as mixtures, and also elucidate biodegradation trends.

For the individual bacteria biodegradation experiments, after 24 hours of growth, 70 mL of the pre-inoculant was transferred into a 500 mL experimental Erlenmeyer flask (working volume of 350 mL), while for the mixed bacteria experiments, equal volumes of each pre-inoculant

individual bacterial growth (after 24 hours) were used to inoculate the experimental flask. The total volume of the mixed bacterial pre-inoculants added to each experimental flask was 70 mL (20% of the total working volume). Our previous work has shown that this approach based on combining equal volumes of different bacterial inocula does not affect the contaminant degradation compared to combining equal cellular concentrations of the different bacteria (Larcher and Yargeau, 2011). The experimental flasks contained the same proportions of MMSM and EE2 as the pre-inoculant flasks. The aliquots of EE2 stock solution (1000 mg/L made in ethanol) added to each experimental flask were based on achieving an initial concentration of 5 mg/L EE2 in a total working volume of 350 mL and assumed that no residual EE2 remained in the pre-inoculant volumes being transferred. However, EE2 present in the pre-inoculant flasks was consistently transferred into the experimental flasks during inoculation, explaining the initial EE2 concentration that was slightly greater than 5 mg/L. The addition of stock EE2 made in 100% ethanol (EtOH) resulted in 0.5% EtOH (v/v) present in the aqeuous matrix. The results of inhibition tests carried out prior to the biodegradation experiments showed that exposure to 0.5% (v/v) EtOH did not inhibit the growth of any of the bacteria being studied. Control experiments conducted with EtOH and MMSM alone confirmed the successful growth of the bacteria in the absence of EE2; it was assumed that the EtOH present in all experiments served as an added carbon source in the aqueous matrix and would not alter the aim of the study investigating the ability of heterotrophic strains and the relative efficacy of individual versus mixed heterotrophic bacteria.

Abiotic control experiments were carried out to verify that the experimental results were due to EE2 biodegradation. Adsorption of EE2 onto each bacteria was found to be within the error of analysis (± 2%) from biodegradation experiments conducted with bacteria killed via autoclaving after pre-inoculation. Although it has been demonstrated by one study that EE2 is predominantly removed via adsorption (Urase and Kikuta, 2005), this study's results are consistent with other previous research where biodegradation was observed to be more important than sorption for

EE2 removal (Andersen et al., 2005), and where less than 5% of the intial EE2 in water was adsorbed onto activated sludge (Ternes et al., 2006).

Analytical methods

Bacterial growth was measured via the optical density at 540 nm using a Thermo Evolution 300 UV-Visible Spectrophotometer; for each measurement 3 mL aliquots from the experimental flask were sampled over time using an automatic pipet with sterile tips. Serial dilutions (up to 10^{-8}) in sterile saline solution (0.85%) were also conducted and plated on BHI agar in order to visually confirm the growth (after 24 to 48 hours incubation at 26°C in the dark) of each of the seven strains of bacteria as well as to ensure there was no contamination.

To monitor the concentration of EE2, 2 mL aliquots were removed from each experimental flask over time and centrifuged at 10,000 rpm for 10 minutes (Thermo IEC MicroCL 21). 1 mL of the supernantant was then syringe-filtered with a 0.22 μm PVDF filter directly into an amber vial for HPLC analysis. Determination of EE2 was carried out using an Agilent 1200 HPLC equipped with a Diode Array Detector (DAD) at a wavelength of 279 nm and a Zorbax Eclipse Plus C18 column (150 x 4.6 mm, 3.5 μm). 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 minutes; the method used a column temperature of 28°C, an injection volume of 50 μL and a flow rate of 0.8 mL/min. The limit of detection and limit of analysis of the HPLC method was 125 μg/L and 417 μg/L, respectively.

Results and Discussion

17α-ethinylestradiol removal by individual bacteria

All seven of the individual bacteria successfully grew in the presence of 17α-ethinylestradiol (EE2); there was no observed effect on bacterial growth due to exposure to the synthetic estrogen. Most of the bacteria grew to a maximum optical density measured at 540 nm ($O.D_{540}$) greater than 2 (2.1 to 2.6), except *R. rhodochrous*, which only reached a maximum $O.D_{540}$ of approximately 1.3. These results are summarized in Table 1 in addition to the maximum percent EE2 removals achieved by each individual bacteria. It is clear that the *Rhodococcus* species were the most successful with 38% to 61% EE2 removal after 300 hours (*R. zopfii*, *R. erythropolis*, *R. equi*) and no detectable EE2 after only 48 hours (*R. rhodochrous*). The impressive EE2 removal achieved by *R. rhodochrous* in less than 48 hours is even more so considering that this bacteria had the lowest maximum $O.D_{540}$, approximately half the cell density attained by the other six bacteria. Experiments conducted with EtOH and MMSM alone (data not shown) showed that *R. rhodochrous* did not use EE2 for growth, which may provide an explanation for the low maximum $O.D_{540}$ value attained by this bacteria. Also, as discussed in the *Biodegradation experiments* section, EtOH acted as an additional carbon source for the bacteria and it may be possible that in the case of *R. rhodochrous* the EtOH caused increased EE2 degradation via co-metabolism. The remaining three bacteria achieved less EE2 removal than the *Rhodococcus* species, ranging from 21% to 34% (*P. putida*, *B. subtilis*, *P. aeruginosa*). The success of the *Rhodococcus* species used in this study compared to the others is not unexpected as this genus is known to be capable of degrading recalcitrant organic compounds with aromatic structures and phenolic moieties like EE2 (Larkin et al., 2005; Martínková et al., 2009). It has also been shown that the presence of EtOH can enhance the hydroxylation of steroid compounds by *Rhodococcus* species (Mutafov et al., 1997) and that the steroid 9α-monooxygenase enzyme, which is produced by *Rhodococcus sp*., can convert steroid compounds into 9α-hydroxy derivatives (BRENDA The Comprehensive Enzyme Information System); the observed success of the *Rhodococcus sp*. in this study may potentially be

attributed to these factors. No previous study has demonstrated comparable removal of EE2 by *R. rhodochrous* , although strains of *R. zopfii* and *R. equi* isolated from activated sludge have demonstrated highly effective EE2-degrading capabilities (70% to 96% removal) (Yoshimoto et al., 2004); and pure cultures of *R. erythropolis* and *R. equi* have also successfully degraded EE2 in the presence of a co-substrate (47% and 39% removal) (O'Grady et al., 2009). The growth curves and corresponding EE2 removals (duplicates for each condition tested) of the two most successful bacterial species from the current study (*R. rhodochrous* and *R. equi*) are illustrated in Figures 1a and 1b.

The experiments conducted with *R. equi* also resulted in the formation of a metabolite that was eluted 0.5 minutes earlier than EE2 during HPLC analysis (Fig. 1b). This by-product of EE2 degradation appeared to fluctuate up and down (represented by the peak area) starting at 24 hours and continuing throughout the experiment (300 hours). Although this oscillation in metabolite peak area seems to be an unsual pattern, it may be a result of two different processes occuring simultaneously: by-product production due to EE2 degradation and consumption by *R. equi*. Previous research has in fact shown that heterotrophic bacteria are capable of degrading both EE2 and its metabolites of degradation (Shi et al., 2004). Recent studies identified two metabolites of EE2-degradation by heterotrophic cultures (4-hydroxy-EE2 and sulfo-EE2), with the latter observed to be resistant to further degradation (Barr et al., 2011; Khunjar et al., 2011). It is possible that the metabolite peak detected due to EE2 degradation by *R. equi* might have consisted of these two metabolites, resulting in the observed oscillation of peak area.

A metabolite formed by both *P. putida* and *R. erythropolis* and eluted at the same HPLC retention time as the one formed by *R. equi* was detected at a slightly different wavelength (274 nm), while *B. subtilis* and *P. aeruginosa* formed a metabolite that eluted 1 minute after EE2 during HPLC analysis and was detected at a wavelength of 230 nm. Several unidentified metabolites of EE2 degradation (by activated sludge samples) have been detected by previous

researchers, with varying polarity relative to EE2 as was observed in this study (Skotnicka-Pitak et al., 2008). No by-products of EE2 degradation by *R. rhodochrous* or *R. zopfii* were detected at any of the wavelengths monitored (210, 220, 230, 240, 250, 274, 279, 284 nm). The lack of soluble metabolite formation detected with the most successful bacterial species (*R. rhodochrous*) may be attributed to the possibility that any metabolites formed were degraded as they were produced, thus preventing accumulation; or the metabolites were formed at concentrations below the limit of detection of the analytical method used.

17α-ethinylestradiol removal by bacterial mixtures

It was anticipated that the mixed consortia of bacteria might be even more successful at degrading EE2 since it is commonly thought that the mineralization of synthetic compounds may be increased by mixing microorganisms together in order to obtain complementary transformation reactions due to the participation of more than one microbial species (Janke and Fritsche, 1985). Although the presence of EtOH in the aqueous matrix prohibited the verification of the extent of mineralization via COD or TOC analysis, this expected trend was not observed in the mixed bacteria experiments with respect to overall EE2 removal. The results demonstrated that the mixed groups of bacteria achieved 42% to 43% EE2 removal which was greater than the removal by three of the individual bacteria (*B. subtilis*: 27%, *P. aeruginosa*: 34%, *P. putida*: 21%), but similar to that of *R. zopfii* and *R. erythropolis* (38% and 45%), and considerably less than those achieved by *R. equi and R. rhodochrous* individually (60% and below the LOD).

The results of the mixed bacteria experiments are shown in Figures 2a and 2b. Figure 2a demonstrates that both bacterial mixtures experienced successful growth reaching a maximum $O.D_{540}$ comparable to the individual bacteria: between 2.1 (Group 2) and 2.6 (Group 1). The overall EE2 removals observed in the two mixtures were virtually identical (Group 1: 43 ± 4%; Group 2: 42 ± 2%), however the rate at which they removed EE2 was different. After 120 hours

Group 2 removed 30% of the initial EE2 compared to 11% removed by Group 1 (Fig. 2b and Table 2). This difference in the rates of EE2 removal may be explained by the different individual bacteria present in the two groups. Group 2 contained all 4 *Rhodococcus* species, which individually obtained the highest EE2 removals throughout the study, while Group 1 only consisted of three. After 120 hours the *Rhodococcus* species removed from 33% EE2 to below the LOD compared with 14% to 26% by the other three strains (*B. subtilis, P. aeruginosa, P. putida*). Of these three non-*Rhodococcus* species the one with the greatest EE2 removal after 120 hours (*B. subtilis*) was present only in Group 2. This resulted in Group 2 having 5 of the most successful individual bacteria versus only 3 in Group 1, which appears to have led Group 2 to remove 3x more EE2 after 120 hours compared to Group 1. However, the EE2 removal rate of Group 1 started to increase resulting in both bacterial mixtures achieving the same overall EE2 removal after 300 hours (Fig. 2b and Table 2). This increased EE2 removal rate observed in Group 1 may again be attributed to the individual bacteria present in the mixture. *P. aeruginosa* achieved the greatest EE2 removal individually in the latter half of the experiment (during the stationary growth phase) from 14% after 120 hours to 34% after 300 hours. This was the only bacteria that did not achieve the majority of its EE2 removal in the first 120 hours (during the exponential growth phase), and it was only present in Group 1. It is likely that the presence of *P. aeruginosa* only in Group 1 is the reason for this observed increase in EE2 removal rate after 120 hours resulting in the EE2 removals achieved by the two mixtures to be the same after 300 hours.

Overall, these results demonstrate that the degradation of EE2 by mixed consortia of bacteria is not always greater than individual bacteria. Also, the combination of individual bacterial species in a mixture can greatly impact the extent and rate of EE2 degradation. The results of this study showed that even a difference of 1 or 2 bacterial species in a mixture can affect the EE2 removal trends observed. This may be the reason for the varied results obtained

277 in past studies ranging from complete EE2 removal (Hashimoto and Murakami, 2009) to none at
278 all (Weber et al., 2005) in different conventional activated sludge samples.
279

280 **Conclusions**

281 The results of the bench-scale experiments demonstrate that the heterotrophic bacteria
282 studied can successfully degrade the synthetic estrogen 17α-ethinylestradiol (EE2). Individually
283 the *Rhodococcus* species were the most successful with up to 61% EE2 removal after 300
284 hours by *R. equi* and no detectable EE2 after only 48 hours by *R. rhodochrous*. These
285 substantial EE2 removals were not equalled by the two mixed bacterial groups studied, which
286 consisted of 5 (Group 1; no *B. subtilis* or *R. zopfii*) and 6 (Group 2; no *P. aeruginosa*) of the
287 above mentioned bacterial species. The average maximum EE2 removals achieved by the
288 mixed groups of bacteria after 300 hours were 43 ± 4% (Group 1) and 42 ± 2% (Group 2).
289 Although the overall EE2 removals of Group 1 and Group 2 were the same, the rates of EE2
290 removal were observed to be different. This illustrates the impact of minor differences in
291 bacterial mixture composition on degradation trends. Importantly, it also demonstrates that the
292 ability of an individual bacteria to degrade a synthetic compound like EE2 does not always
293 represent what will occur when it is combined with other bacterial cultures to form mixed
294 consortia.
295 These observations help explain the variable EE2 removals by different activated sludge
296 samples reported in literature. They also indicate that during biological wastewater treatment
297 (activated sludge) the removal of EE2 and similar compounds may be due to the activity of
298 heterotrophs and may not rely solely on nitrifying bacteria as previously suggested in literature.
299 Future work should examine the capabilities of the successful bacteria identified in this study to
300 degrade lower concentrations of EE2 in wastewater samples; this would explore the
301 degradation trends under conditions that more closely simulate wastewater treatment.

Acknowledgements

This work was funded by the McGill Engineering Doctoral Award (MEDA), the J.W. McConnell Foundation, the Natural Sciences and Engineering Research Council of Canada (NSERC) and Health Canada.

References

BRENDA The Comprehensive Enzyme Information System (http://www.brenda-enzymes.org/index.php4).

Andersen, H.R., Hansen, M., Kjlholt, J., Stuer-Lauridsen, F., Ternes, T., Halling-Srensen, B., 2005. Assessment of the importance of sorption for steroid estrogens removal during activated sludge treatment. Chemosphere 61, 139-146.

Aravindakshan, J., Gregory, M., Marcogliese, D.J., Fournier, M., Cyr, D.G., 2004. Consumption of Xenoestrogen-Contaminated Fish during Lactation Alters Adult Male Reproductive Function. Toxicological Sciences 81, 179-189.

Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., Samperi, R., 2000. Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water. Environmental Science & Technology 34, 5059-5066.

Barr, W.J., Yi, T., Aga, D., Acevedo, O., Harper, W.F., 2011. Using Electronic Theory To Identify Metabolites Present in 17α-Ethinylestradiol Biotransformation Pathways. Environmental Science & Technology 46, 760-768.

Carlsson, C., Johansson, A.-K., Alvan, G., Bergman, K., Kuhler, T., 2006. Are pharmaceuticals potent environmental pollutants?. Part I: Environmental risk assessments of selected active pharmaceutical ingredients. Science of the Total Environment 364, 67-87.

Cevasco, A., Urbatzka, R., Bottero, S., Massari, A., Pedemonte, F., Kloas, W., Mandich, A., 2008. Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of Xenopus laevis: II. Effects on gonad histomorphology. Comp Biochem Physiol C Toxicol Pharmacol 147, 241-251.

Cicek, N., Londry, K., Oleszkiewicz, J.A., Wong, D., Lee, Y., 2007. Removal of Selected Natural and Synthetic Estrogenic Compounds in a Canadian Full-Scale Municipal Wastewater Treatment Plant. Water Environment Research 79, 795-800.

Clouzot, L., Doumenq, P., Roche, N., Marrot, B., 2010. Kinetic parameters for 17α-ethinylestradiol removal by nitrifying activated sludge developed in a membrane bioreactor. Bioresource Technology 101, 6425-6431.

Clouzot, L., Marrot, B., Doumenq, P., Roche, N., 2008. 17-ethinylestradiol: An endocrine disrupter of great concern. Analytical methods and removal processes applied to water purification. A review. Environmental Progress 27, 383-396.

Daughton, C.G., 2003. Chemicals from pharmaceuticals and personal care products, in: Dasch, J. (Ed.), Water: Sciences and Issues. MacMillan Reference, New York.

De Gusseme, B., Pycke, B., Hennebel, T., Marcoen, A., Vlaeminck, S.E., Noppe, H., Boon, N., Verstraete, W., 2009. Biological removal of 17alpha-ethinylestradiol by a nitrifier enrichment culture in a membrane bioreactor. Water Res 43, 2493-2503.

Gaulke, L.S., Strand, S.E., Kalhorn, T.F., Stensel, H.D., 2008. 17alpha-ethinylestradiol transformation via abiotic nitration in the presence of ammonia oxidizing bacteria. Environ Sci Technol 42, 7622-7627.
Gomes, R.L., Scrimshaw, M.D., Lester, J.N., 2003. Determination of endocrine disrupters in sewage treatment and receiving waters. TRAC Trends in Analytical Chemistry 22, 697-707.
Hannah, R., D'Aco, V., Anderson, P., Buzby, M., Caldwell, D., Cunningham, V., Ericson, J., Johnson, A., Parke, N., Samuelian, J., Sumpter, J., 2009. Exposure assessment of 17a-ethinylestradiol in surface waters of the United States and Europe. Environmental Toxicology and Chemistry 28, 2725.
Hashimoto, T., Murakami, T., 2009. Removal and degradation characteristics of natural and synthetic estrogens by activated sludge in batch experiments. Water Research 43, 573-582.
Howdeshell, K.L., Furr, J., Lambright, C.R., Wilson, V.S., Ryan, B.C., Gray, L.E., Jr., 2008. Gestational and Lactational Exposure to Ethinyl Estradiol, but not Bisphenol A, Decreases Androgen-Dependent Reproductive Organ Weights and Epididymal Sperm Abundance in the Male Long Evans Hooded Rat. Toxicological Sciences 102, 371-382.
Janke, D., Fritsche, W., 1985. Nature and significance of microbial cometabolism of xenobiotics. Journal of Basic Microbiology 25, 603-619.
Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C.R., van Aerle, R., Santos, E., Brighty, G., 2006. Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. Environ Health Perspect 114 Suppl 1, 32-39.
Johnson, A.C., Sumpter, J.P., 2001. Removal of Endocrine-Disrupting Chemicals in Activated Sludge Treatment Works. Environmental Science & Technology 35, 4697-4703.
Johnson, A.C., Williams, R.J., 2004. A Model To Estimate Influent and Effluent Concentrations of Estradiol, Estrone, and Ethinylestradiol at Sewage Treatment Works. Environmental Science & Technology 38, 3649-3658.
Joss, A., Keller, E., Alder, A.C., Göbel, A., McArdell, C.S., Ternes, T., Siegrist, H., 2005. Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Research 39, 3139-3152.
Khunjar, W.O., Mackintosh, S.A., Skotnicka-Pitak, J., Baik, S., Aga, D.S., Love, N.G., 2011. Elucidating the Relative Roles of Ammonia Oxidizing and Heterotrophic Bacteria during the Biotransformation of 17α-Ethinylestradiol and Trimethoprim. Environmental Science & Technology 45, 3605-3612.
Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environmental Science and Technology 36, 1202-1211.
Lai, K.M., Johnson, K.L., Scrimshaw, M.D., Lester, J.N., 2000. Binding of Waterborne Steroid Estrogens to Solid Phases in River and Estuarine Systems. Environmental Science and Technology 34, 5.
Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. The Effects of Natural and Synthetic Steroid Estrogens in Relation to their Environmental Occurrence. Critical Reviews in Toxicology 32, 113.
Lange, A., Paull, G.C., Coe, T.S., Katsu, Y., Urushitani, H., Iguchi, T., Tyler, C.R., 2009. Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. Environ Sci Technol 43, 1219-1225.
Larcher, S., Delbes, G., Robaire, B., Yargeau, V., 2012. Degradation of 17alpha-ethinylestradiol by ozonation - identification of the by-products and assessment of their estrogenicity and toxicity. Environment International 39, 66-72.
Larcher, S., Yargeau, V., 2011. Biodegradation of sulfamethoxazole by individual and mixed bacteria. Applied Microbiology and Biotechnology 91, 211-218.

Larkin, M.J., Kulakov, L.A., Allen, C.C.R., 2005. Biodegradation and Rhodococcus - masters of catabolic versatility. Current Opinion in Biotechnology 16, 282-290.
Martínková, L., Uhnáková, B., Pátek, M., Nesvera, J., Kren, V., 2009. Biodegradation potential of the genus Rhodococcus. Environment International 35, 162-177.
Martinović, D., Hogarth, W.T., Jones, R.E., Sorensen, P.W., 2007. Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. Environmental Toxicology and Chemistry 26, 271-278.
Miège, C., Choubert, J.M., Ribeiro, L., Eusèbe, M., Coquery, M., 2009. Fate of pharmaceuticals and personal care products in wastewater treatment plants - conception of a database and first results. Environmental Pollution 157, 1721-1726.
Mutafov, S., Angelova, B., Avramova, T., Boyadjieva, L., Dimova, I., 1997. The inducibility of 9α-steroid hydroxylating activity in resting Rhodococcus sp. cells. Process Biochemistry 32, 585-589.
O'Grady, D., S., E., Yargeau, V., 2009. Removal of Aqueous 17a-Ethinylestradiol by *Rhodococcus* Specis. Environmental Engineering Science 26, 1393-1400.
Pauwels, B., Wille, K., Noppe, H., De Brabander, H., Van De Wiele, T., Verstraete, W., Boon, N., 2008. 17-ethinylestradiol cometabolism by bacteria degrading estrone, 17-estradiol and estriol. Biodegradation 19, 683-693.
Petrovic, M., Sole, M., Lopez de Alda, M.J., Barcelo, D., 2002. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environmental Toxicology and Chemistry 21, 2146-2156.
Rani, A., Porwal, S., Sharma, R., Kapley, A., Purohit, H.J., Kalia, V.C., 2008. Assessment of microbial diversity in effluent treatment plants by culture dependent and culture independent approaches. Bioresource Technology 99, 7098-7107.
Ren, Y.X., Nakano, K., Nomura, M., Chiba, N., Nishimura, O., 2007. Effects of bacterial activity on estrogen removal in nitrifying activated sludge. Water Res 41, 3089-3096.
Seviour, R., Kragelund, C., Kong, Y., Eales, K., Nielsen, J., Nielsen, P., 2008. Ecophysiology of the Actinobacteria in activated sludge systems. Antonie van Leeuwenhoek 94, 21-33.
Shi, J., Fujisawa, S., Nakai, S., Hosomi, M., 2004. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. Water Research 38, 2323-2330.
Silva, C.P., Otero, M., Esteves, V., 2012. Processes for the elimination of estrogenic steroid hormones from water: A review. Environmental Pollution 165, 38-58.
Skotnicka-Pitak, J., Garcia, E.M., Pitak, M., Aga, D.S., 2008. Identification of the transformation products of 17-ethinylestradiol and 17-estradiol by mass spectrometry and other instrumental techniques. TrAC - Trends in Analytical Chemistry 27, 1036-1052.
Skotnicka-Pitak, J., Khunjar, W.O., Love, N.G., Aga, D.S., 2009. Characterization of metabolites formed during the biotransformation of 17-ethinylestradiol by Nitrosomonas europaea in batch and continuous flow bioreactors. Environmental Science and Technology 43, 3549-3555.
Ternes, T., Janex-Habibi, M.-L., Knacker, T., Kreuzinger, N., Siegrist, H., 2006. Assessment of technologies for the removal of pharmaceuticals and personal care products in sewage and drinking water facilities to improve the indirect potable water reuse (POSEIDON Final Report-EU Research Programme, http://poseidon.bafg.de/servlet/is/2888/).
Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Research 32, 3245-3260.
Ternes, T.A., Kreckel, P., Mueller, J., 1999a. Behaviour and occurrence of estrogens in municipal sewage treatment plants -- II. Aerobic batch experiments with activated sludge. The Science of The Total Environment 225, 91-99.

444 Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M., 1999b. Behavior
445 and occurrence of estrogens in municipal sewage treatment plants -- I. Investigations in
446 Germany, Canada and Brazil. The Science of The Total Environment 225, 81-90.
447 Urase, T., Kikuta, T., 2005. Separate estimation of adsorption and degradation of
448 pharmaceutical substances and estrogens in the activated sludge process. Water Research
449 39, 1289-1300.
450 Vader, J.S., van Ginkel, C.G., Sperling, F.M.G.M., de Jong, J., de Boer, W., de Graaf, J.S., van
451 der Most, M., Stokman, P.G.W., 2000. Degradation of ethinyl estradiol by nitrifying activated
452 sludge. Chemosphere 41, 1239-1243.
453 Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O., 2008.
454 Reproductive Disruption in Fish Downstream from an Estrogenic Wastewater Effluent.
455 Environmental Science & Technology 42, 3407-3414.
456 Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambré, C., Tarazona, J.V., Brandt, I.,
457 Vethaak, A.D., 2000. Health Effects of Endocrine-Disrupting Chemicals on Wildlife, with
458 Special Reference to the European Situation. Critical Reviews in Toxicology 30, 71-133.
459 Weber, S., Leuschner, P., Kämpfer, P., Dott, W., Hollender, J., 2005. Degradation of estradiol
460 and ethinyl estradiol by activated sludge and by a defined mixed culture. Applied
461 Microbiology and Biotechnology 67, 106-112.
462 Yi, T., Harper, W.F., Jr., 2007. The link between nitrification and biotransformation of 17alpha-
463 ethinylestradiol. Environ Sci Technol 41, 4311-4316.
464 Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., Kimura, K.,
465 Saino, H., Sawada, H., Omura, H., 2004. Degradation of Estrogens by Rhodococcus zopfii
466 and Rhodococcus equi Isolates from Activated Sludge in Wastewater Treatment Plants.
467 Applied and Environment Microbiology 70, 5283-5289.

Table 1 Summary of individual bacteria results: maximum growth (optical density measured at 540 nm) and percent EE2 removal after 300 hours.

Bacteria	Maximum Growth ($O.D_{540}$)[a]	EE2 percent removal[b]	Metabolite Detection?[c]
B. subtilis	2.12, 2.13	27% ± 2%	Yes (230 nm)
P. aeruginosa	2.57, 2.61	34% ± 2%	Yes (230 nm)
P. putida	2.32, 2.35	21% ±2%	Yes (274 nm)
R. equi	2.23, 2.24	61 %± 1 %	Yes (279 nm)
R. erythropolis	2.00, 2.02	46% ± 2%	Yes (274 nm)
R. rhodochrous	1.20, 1.30	no EE2 detected after 48hrs	No
R. zopfii	2.26, 2.25	38% ± 1%	No

[a] maximum optical density values measured at 540 nm ($O.D_{540}$) for each duplicate experiment

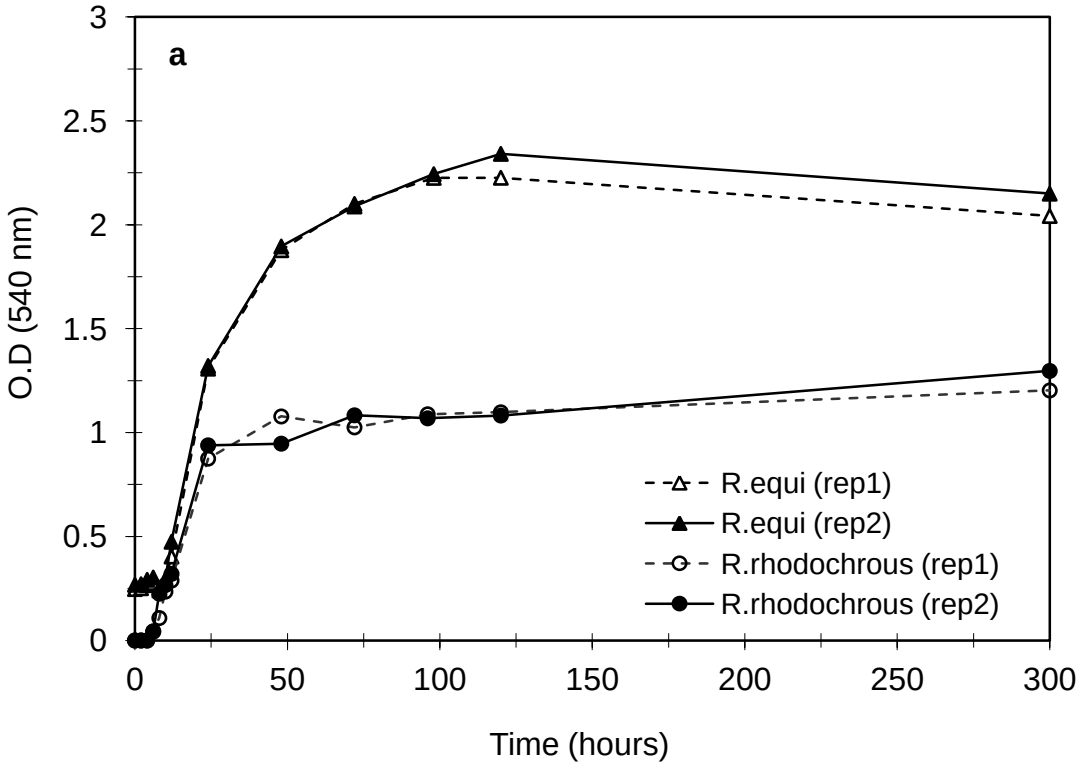
[b] average EE2 percent removal of duplicate experiments ± range in values (after 300 hours)

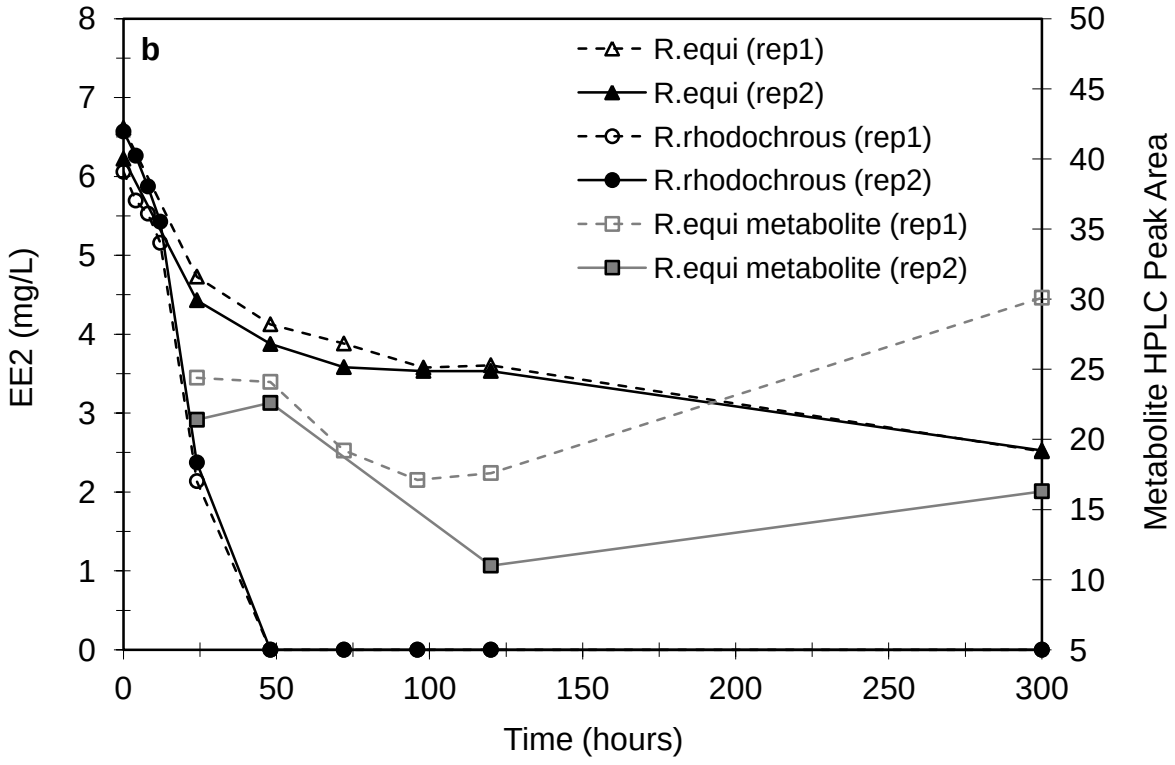
[c] wavelength of metabolite detection listed in brackets

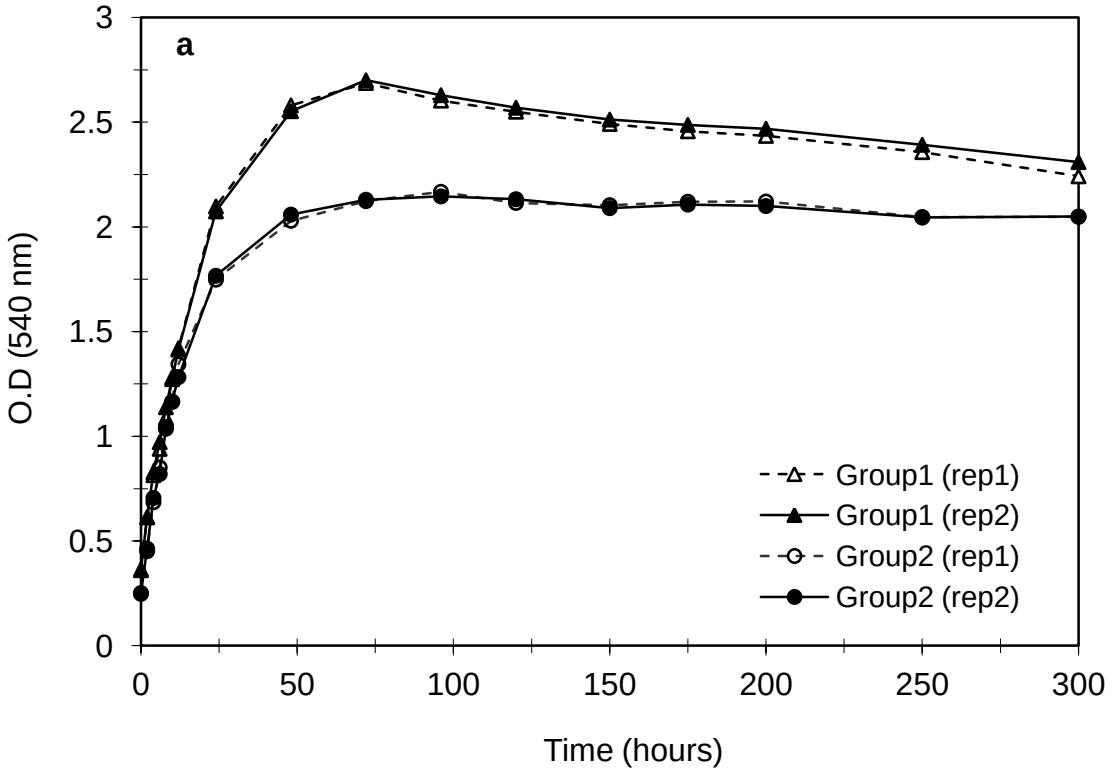
Table 2 Summary of the percent EE2 removals achieved after 120 hours and 300 hours by the bacterial mixtures: Group 1 (*P. aeruginosa, P. putida, R. equi, R. erythropolis and R. rhodochrous*) and Group 2 (*B. subtilis, P. putida, R. equi, R. erythropolis, R. rhodochrous, and R. zopfii*).

	EE2 percent removal[a]	
	120 hrs	300 hrs
Group 1	11% ± 1%	43% ± 4%
Group 2	30% ± 1%	42% ± 2%

[a] average of duplicate experiments ± range in values


a
O.D (540 nm)
Time (hours)
R.equi (rep1)
R.equi (rep2)
R.rhodochrous (rep1)
R.rhodochrous (rep2)


b
EE2 (mg/L)
Metabolite HPLC Peak Area
Time (hours)
R.equi (rep1)
R.equi (rep2)
R.rhodochrous (rep1)
R.rhodochrous (rep2)
R.equi metabolite (rep1)
R.equi metabolite (rep2)


a
O.D (540 nm)
Time (hours)
Group1 (rep1)
Group1 (rep2)
Group2 (rep1)
Group2 (rep2)
0
0.5
1
1.5
2
2.5
3
0
50
100
150
200
250
300

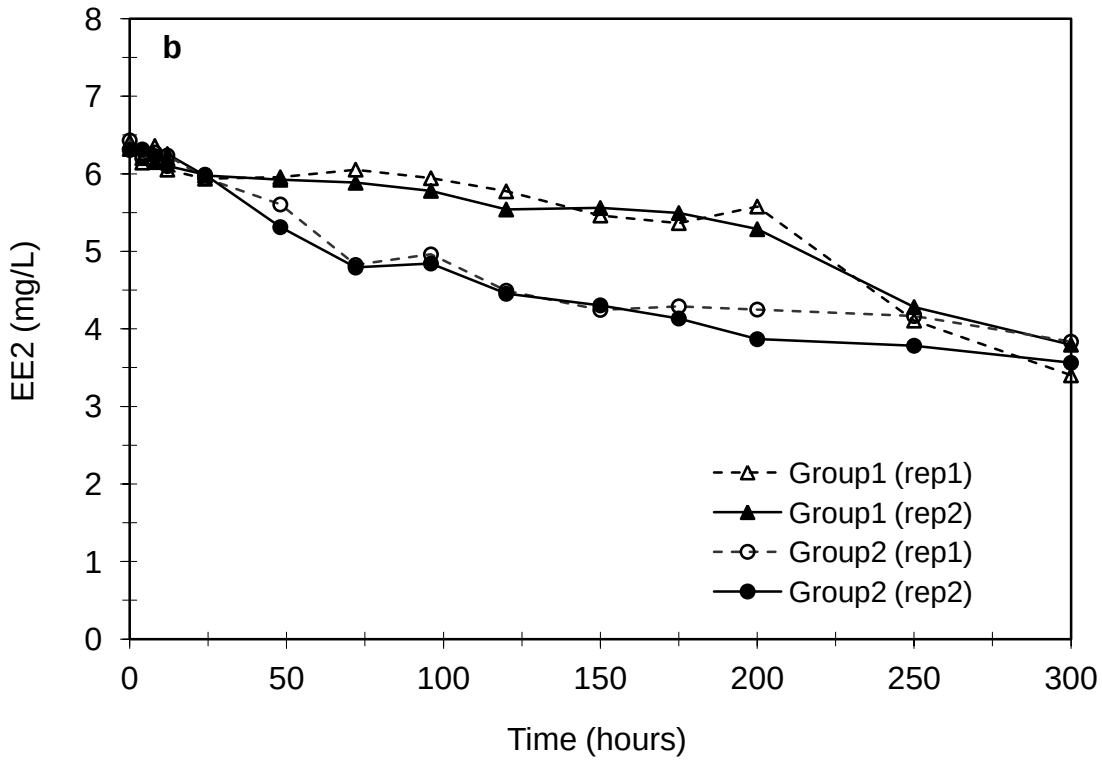

b
EE2 (mg/L)
Time (hours)
Group1 (rep1)
Group1 (rep2)
Group2 (rep1)
Group2 (rep2)
0
1
2
3
4
5
6
7
8
0
50
100
150
200
250
300

Fig. 1. Biodegradation of 5 mg/L 17α-ethinylestradiol (EE2) by individual *Rhodococcus* species (*R. equi* and *R. rhodochrous*). (a) Bacterial growth; (b) removal of EE2 over 300 hours.

Fig. 2. Biodegradation of 5 mg/L 17α-ethinylestradiol (EE2) by mixed bacteria 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*). (a) Bacterial growth; (b) removal of EE2 over 300 hours.