Biodegradation of sulfamethoxazole by individual and mixed bacteria

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Abstract Antibiotic compounds, like sulfamethoxazole (SMX), have become a concern in the aquatic environment due to the potential development of antibacterial resistances. Due to excretion and disposal SMX has been frequently detected in wastewaters and surface waters. SMX removal in conventional wastewater treatment plants (WWTPs) ranges from 0 to 90% and there are opposing results regarding its biodegradability at lab-scale. The objective of this research was to determine the ability of pure cultures of individual and mixed consortia of bacteria (*B. subtilis, P. aeruginosa, P. putida, R. equi, R. erythropolis, R. rhodocrous, and R. zopfii*) known to exist in WWTP activated sludge to remove SMX. Results showed that *R. equi* alone had the greatest ability to remove SMX leading to 29% removal (with glucose) and the formation of a metabolite. Degradation pathways and metabolite structures have been proposed based on the potential enzymes produced by *R. equi*. When *R. equi* was mixed with other microorganisms a positive synergistic effect was not observed and the maximum SMX removal achieved was 5%. This indicates that pure culture results cannot be extrapolated to mixed culture conditions and the methodology developed here to study the biodegradability of compounds under controlled mixed culture conditions offers an alternative to conventional studies using pure bacterial cultures or inocula from activated sludge sources consisting of unknown and variable microbial populations.

Keywords: sulfamethoxazole (SMX), antibiotics, biodegradation, *R. equi*, mixed cultures

Introduction

The presence of pharmaceutical compounds in the environment has become an increasingly important issue due to their widespread use and disposal worldwide. Antibiotic compounds represent one of the largest concerns as it is believed their presence will lead to antibacterial resistances in bacteria present in the environment and in biological wastewater treatment (activated sludge) (Andersson 2003; Costanzo et al. 2005; Daughton 2003; Daughton and Ternes 1999; Goni-Urriza et al. 2000; Jorgensen and Halling-Sorensen 2000; Reinthaler et al. 2003; Volkmann et al. 2004).

The estimated antibiotic consumption worldwide ranges from 100,000 to 200,000 tons annually, and due to excretion (which can reach up to 90% of the ingested amount both as the original parent compound or as metabolites) and disposal, up to μg/L levels have been detected in raw wastewater (Hirsch et al. 1999; Kümmerer 2009a, 2009b, 2009c; Perez et al. 2005). The synthetic compound sulfamethoxazole (SMX) is one of the most commonly used antibiotics (Cavallucci 2007), and has thus been frequently detected in wastewaters at up to μg/L levels and surface waters at ng/L levels (Batt et al. 2007; Benotti et al. 2009; Joss et al. 2005; Kolpin et al. 2002; Miège et al. 2009; Peng et al. 2006; Yargeau et al. 2007).

SMX removal rates in conventional wastewater treatment plants (WWTPs) have been measured to range from 0 to 90% (Carballa et al. 2004; Jones 2005; Miège et al. 2009; Ternes 2006a; Xu et al. 2007) with up to an estimated 60% removal achieved during the activated

sludge treatment step (Göbel et al. 2007). At lab-scale there have been mixed results regarding the biodegradation of SMX from significant removal in activated sludge (Perez et al. 2005) to minimal biodegradation (Joss et al. 2006). There are also contradictory results regarding the effect of the presence of a more readily degradable carbon source, from an increase of over 30% SMX removal using the OECD Closed Bottle Test (Alexy et al. 2004) to no SMX removal with added carbon and nitrogen using a lab-scale sequencing batch reactor (Drillia et al. 2005).

The aim of this research was to determine the ability of pure cultures of individual bacteria that have been found in activated sludge (Benedict and Carlson 1971; Rani et al. 2008; Seviour et al. 2008) as well as controlled mixtures of these pure bacterial cultures to degrade SMX in the presence and absence of a readily degradable carbon source (glucose).

Materials and Methods

Chemicals

All chemicals used were either reagent grade or HPLC grade. The SMX (CAS 723-46-46), ammonium nitrate (NH_4NO_3), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and glucose assay kit (GAHK-20) 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, phosphoric acid and acetonitrile (ACN) were purchased from Fisher Scientific, Canada. The McFarland Standard #2 was obtained from Biomérieux, France; the BHI (Brain Heart Infusion) Agar was obtained from Becton-Dickinson & Co., Canada; and glucose was obtained from A & C American Chemicals Ltd, Canada.

Bacteria and cultivation

The pure bacterial cultures used in this study were obtained from Cedarlane® Canada and stored at -80°C in a glycerol/BHI mixture. Each bacteria was thawed and grown individually in BHI broth for 24 hours in the dark 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 investigated were (ATCC#): *Bacillus subtilis* (6051), *Pseudomonas aeruginosa* (PA01), *Pseudomonas putida* (12633), *Rhodoccocus equi* (13557), *Rhodoccocus erythropolis* (4277), *Rhodoccocus rhodocrous* (13808), and *Rhodoccocus zopfii* (51349).

Inhibition tests

Prior to the mixed bacteria experiments, inhibition tests were conducted to determine if all seven bacteria would grow together succesfully. The results of these tests determined the final bacterial mixtures studied.

For each bacteria, the growth on BHI agar plates was used to make cellular standards (McFarland Standard #2 ; $3x10^8$ cells/mL) in 6 mL of a 0.85% sterile saline solution (8.5 g/L sodium chloride). Using an automatic micropipet with sterile disposable tips, 100 μL of each bacterial standard solution was dispensed onto separate BHI agar plates and then spread evenly using sterile swabs. Sterile filter papers (8mm diameter) were soaked in the other six individual bacterial standard solutions and placed onto the plates (spaced evenly apart) using sterilized tweezers. The plates were incubated in the dark for 24-48 hours at 26°C and visually monitored to determine any bacterial inhibition.

Biodegradation experiments

Biodegradation experiments were carried out in duplicate using each of the seven bacteria individually and the two bacterial mixtures (Group 1 and Group 2) determined via inhibition tests and outlined in the Results section. Again for each individual bacteria, growth on BHI agar at 26°C was used to make cellular standards (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), 6 mg/L SMX and 0.5 g/L glucose (in the cases considering an easily degradable carbon source) 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), and yeast extract (0.6 g per liter of the working volume).

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 500 mL experimental erlenmeyer flask (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). It should be noted that although equal volumes of each bacterial pre-inoculant were mixed together, since each individual bacteria experienced different rates of growth during the 24 hour pre-inoculation the number of cells per volume were not equal. In order to determine whether

this unequal mixing of the bacterial cells had a significant impact on the observed results, additional experiments were carried out in duplicate using bacterial pre-inoculants diluted with sterile MMSM to achieve an identical number of cells in the mixtures tested. The experimental flasks contained the same proportions of MMSM, SMX, and glucose (if necessary) as the pre-inoculant flasks. SMX present in the pre-inoculant flasks was transferred into the experimental flasks during inoculation, explaining the initial SMX concentration that was slightly higher than 6 mg/L.

Control experiments were carried out with the individual and mixed bacteria to verify that any metabolites detected were due solely to SMX transformation. These controls were done by conducting the above described biodegradation experiments in MMSM ± glucose without SMX. Adsorption onto each bacteria was found to be insignificant as indicated by experiments conducted with bacteria killed via autoclaving immediately after pre-inoculation.

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 SMX, 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 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 x 4.6 mm, 3.5 μm). 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 minutes; the method used a column temperature of 40°C, an injection volume of 10 μL and a flow rate of 0.7 mL/min. The limit of detection and limit of quantification of the HPLC method was 2 μg/L and 7 μg/L respectively while the measurement uncertainty was 2% (based on the variation of SMX standard measurements).

Results

Sulfamethoxazole removal by individual bacteria

All seven of the individual bacteria grew in the presence of sulfamethoxazole (SMX), therefore there was no inhibition of bacterial growth observed due to exposure to the antibiotic compound. In the presence of glucose, most of the bacteria grew to a maximum optical density measured at 540 nm ($O.D_{540}$) greater than 1, except *B. subtilis*, which only reached a maximum $O.D_{540}$ of approximately 0.4. Both *R. equi* and *R. zopfii* exhibited the greatest growth in the presence of glucose, reaching a maximum $O.D_{540}$ of approximately 1.3. In the absence of glucose, the maximum $O.D_{540}$ of most of the bacteria was in the range of 0.5 to 0.6, except *B. subtilis* (0.28) and *R. rhodocrous* (0.8). These results are summarized in Table 1 and demonstrate that the presence of glucose resulted in an average increase in bacterial cell density of 114% (minimum = 54%, maximum = 170%). Glucose measurements indicate that after 120 hours there was approximately 1.3% to 2.2% of the initial 0.5 g/L remaining in solution.

Based on the successful growth of almost all of the bacteria, it was expected that the corresponding SMX removals would be considerable. However, the only significant removal of SMX after 120 hours occurred in the presence of *R. equi* (approximately 15% without glucose; 29% with glucose) while the SMX removal achieved by the other six individual bacteria ranged from 0 to 6.6%. Considering the measurement uncertainty (2%) the majority of the bacteria were not effective at removing SMX. The growth curves (duplicates for each condition tested) of *R. equi* and corresponding SMX removals are illustrated in Figure 1a and 1b, and a summary of all the results of the individual bacteria experiments is outlined in Table 1.

The experiments conducted with *R. equi* resulted in the formation of a metabolite (in the presence of glucose) that was eluted 0.3 minutes earlier than SMX during HPLC analysis (Fig. 1b); this was also observed to occur in the experiments carried out using *P. aeruginosa* both with and without glucose (Table 1).

Determination of mixed bacterial groups

As described in the Materials & Methods section, inhibition tests were carried out with all seven individual bacteria to determine if they could successfully grow together. Visual observations of the growth of bacteria on the BHI agar plates demonstrated that *P. aeruginosa* inhibited the growth of *B. subtilis* and *R. zopfii* (i.e. on the plates covered evenly with *B. subtilis* and *R. zopfii*, rings of "no growth" were seen around the filter papers soaked in *P. aeruginosa* standard). Based on the inhibition test results, two different bacterial mixtures were studied. Group 1 consisted of *P. aeruginosa, P. putida, R. equi, R. erythropolis and R. rhodocrous*; and Group 2 consisted of *B. subtilis, P. putida, R. equi, R. erythropolis, R. rhodocrous, and R. zopfii*.

Sulfamethoxazole removal by bacterial mixtures

The results of the mixed bacteria experiments are shown in Figures 2a to 2d. Figures 2a (Group 1) and 2b (Group 2) show that both bacterial mixtures experienced similar growth reaching a maximum $O.D_{540}$ of approximately 1.2 (SMX + glucose) and 0.65 (SMX alone). This demonstrates the same trend observed with the individual bacteria of increased cell density in the presence of glucose. Similar to the individual bacteria experiments the initial 0.5 g/L glucose was not completely consumed; 7% to 10% remained after 120 hours and 1.6% to 2.2% remained after 300 hours.

In Group 1 (Fig. 2c), up to 5% SMX removal occurred in the presence of glucose and there was also the formation of a metabolite which was eluted at the same retention time during HPLC analysis as the by-product formed during SMX removal by *R. equi* (Fig. 1b) and *P. aeruginosa* (Table 1). In the absence of glucose, this removal was unchanged but no metabolite was formed. Experiments conducted with MMSM + glucose alone confirmed this metabolite resulted from SMX and not glucose degradation. These results demonstrate that, overall, there was minimal SMX removal via biodegradation (in the absence and presence of glucose) by the mixed bacteria in Group 1.

In Group 2 (Fig. 2d), the maximum SMX removal was approximately 5% (in the presence of glucose); without glucose, the SMX removal was halved. There was no metabolite formation observed with this mixture of bacteria with or without glucose.

R. equi, P. aeruginosa and P. putida were consistently the fastest growing bacteria during pre-inoculation and therefore were the dominant microorganisms in each mixed group. As discussed previously additional experiments were carried out using bacterial pre-inoculants diluted with

sterile MMSM in order to achieve an identical number of cells of the 5 bacteria in Group 1 and the 6 bacteria in Group 2. The results of these experiments (Table 2, supplemental) show that diluting the bacterial pre-inoculant growths prior to mixing them did not significantly alter the trends previously observed. The maximum growth (measured via $O.D_{540}$) achieved by both mixed groups was virtually identical regardless of the pre-inoculant dilution, and in the presence of glucose the SMX removal was approximately 5% for both Group 1 and Group 2 (with the formation of a by-product observed only in undiluted Group 1). In the absence of glucose, the dilution slightly lowered the SMX removal observed by 1.6% (Group 1) and 1.1% (Group 2) which is within the uncertainty in measurement (2%) therefore there was essentially no change resulting from pre-inoculant dilution.

Discussion

Individual bacteria

The results of the SMX degradation experiments carried out using individual bacterial species common to activated sludge (*B. subtilis, P. aeruginosa, P. putida, R. equi, R. erythropolis, R. rhodocrous, R. zopfii*), demonstrated that there was only slight SMX removal by 6 of the 7 bacteria studied (0 to 6.6%) (Table 1). This agrees with previously published results demonstrating that SMX is not readily biodegradable via studies using the OECD Closed-Bottle, Zahn-Wellens, and CO_2 evolutions tests (Al-Ahmad et al. 1999; Gartiser et al. 2007) as well as lab-scale experiments using inocula from activated sludge sources (Joss et al. 2006). *R. equi* was the only bacteria observed to effectively remove SMX from 15% up to 29% with glucose addition (Fig. 1a and 1b). The presence of glucose not only increased the growth of *R. equi* by 125%, resulting in almost twice as much SMX removal, but also resulted in the formation of a

degradation by-product. Although it appears that there is a direct relationship between the presence of glucose leading to increased cell density and SMX removal, further inspection of the results in Table 1 show that while there was an average 114% increase in cell density due to the presence of glucose for all the individual bacteria experiments, only three of these resulted in increased SMX removal (*R. equi, P. aeruginosa, B. subtilis*). In addition, *P. putida* exhibited the largest increase in cell density due to the presence of glucose (170%), yet there was no detectable SMX removal with or without glucose. Increased bacterial growth in the presence of glucose without a correspoding increase in SMX removal may be a result of these bacteria preferentially consuming this readily degradable carbon source as has been observed to occur in previous research with activated sludge inoculum (Drillia et al. 2005). *P. aeruginosa* was the only other bacteria whose SMX degradation resulted in the formation of a metabolite both with and without glucose; the 130% increase in cell density due to glucose resulted in over three times more SMX removal and increased metabolite formation (HPLC peak area doubled). It should be noted that control experiments were conducted as described previously ensuring that the metabolites formed resulted from SMX transformation.

It is known that the genus *Rhodococcus* consists of bacteria capable of degrading organic compounds that are not easily biodegraded and that bacteria in the genus *Pseudomonas* can have similar capabilities (Larkin et al. 2005; Martínková et al. 2009). Therefore, the success of *R. equi* and *P. aeruginosa* are not entirely unexpected but the reasons for their success compared to the other individual bacteria are not clear. It is believed that the degradation of SMX and formation of metabolites is due to the enzymes produced by these bacteria which are not produced to a great extent by the other individual bacteria studied. Using the Enzyme Database BRENDA (BRENDA The Comprehensive Enzyme Information System) the different enzymes

produced by the bacteria and their characteristics were determined. It was discovered that arylamine N-acetyltransferase is produced by *Pseudomonas aeruginosa* and *Rhodococcus* species in general and that this enzyme has a specificity for aromatic amines and can use SMX as a substrate. It is hypothesized that the *R. equi* strain used in this study produced more arylamine N-acetyltransferase than any of the other *Rhodococcus* species tested resulting in higher SMX removal. Although this hypothesis may explain why *R. equi* was the most successful *Rhodococcus* species, it does not explain why *P. aeruginosa* had less SMX removal (up to 5.6%) while still resulting in metabolite formation. This may be due to another enzyme produced by *P. aeruginosa*: dihydropteroate synthetase (DHPS). The antibiotic function of SMX is the competitive inhibition of DHPS, which interrupts the formation of folic acid from 4 amino-benzoic acid in the human body. Since *P. aeruginosa* produces DHPS, it will compete with the arylamine N-acetyltransferase that is also being produced, which may have resulted in the observed low SMX removal. While arylamine N-acetyltransferase attacks the aromatic amine of SMX producing a by-product, the DHPS binds to SMX. This may account for the detection of a tiny by-product by *P. aeruginosa* even though less SMX removal was observed. The proposed metabolite would have a structure containing an acetyl group attached to the aromatic amine in SMX.

It is also possible that additional enzymes produced by *R. equi* could lead to the hydrolysis of the acetylated SMX by-product. It has been found that a strain of *R. equi* was capable of producing an amidase that degraded lysergamide to lysergic acid (Martínková et al. 2000) and this may have occurred to the acetylated metabolite of SMX to form an alcohol derivative. *R. equi* can also produce urethanase, which hydrolyzes anilides, and N-acetyl-phenylethylamine hydrolase which hydrolizes N-acetylated compounds (BRENDA The Comprehensive Enzyme

Information System). The increased growth of *R. equi* in the presence of glucose may have lead to the increased production of these enzymes and the formation of an alcohol derivative of the acetylated metabolite. The similarity in structure between the acetylated metabolite and its alcohol derivative would have resulted in similar HPLC elution times, explaining the retention time of the by-products that was 0.3 minutes earlier than that of SMX. Also, both of these proposed metabolites are more polar than SMX, which would lead to the observed elution earlier than SMX during HPLC analysis. Figure 3 (supplemental) illustrates the structure of SMX and the proposed acetylated metabolite and its alcohol derivative.

Mixed bacteria

As a result of the inhibition tests the mixed consortia studied consisted of two different groups of bacteria. It was anticipated that the mixed consortia of bacteria might be more successful at degrading SMX as it is commonly thought that synthetic chemicals resistant to degradation by an individual microorganism may be mineralized via complementary transformation reactions due to the participation of more than one microbial species (Janke and Fritsche 1985). However, this expected trend was not observed in the mixed bacteria experiments even after 300 hours (Fig. 2c and 2d). In fact the results demonstrated that *R. equi* alone was more successful (in less than half the time) than either group of mixed bacteria at removing sulfamethoxazole in the presence and absence of glucose. Overall, whether or not equal cellular concentrations of bacterial solutions were combined to form a mixed group of bacteria (Table 2), the maximum removal of SMX achieved was 5% after 300 hours. This is poor compared to the results of *R. equi* alone, which

achieved 5% SMX removal after only 24 hours and 15% (without glucose) to 29% (with glucose) removal after 120 hours.

It should be noted that the mixed bacterial group in which a metabolite was detected (Group 1) contained both species which produced the metabolite individually (*P. aeruginosa* and *R. equi*) whereas Group 2, in which no metabolite was detected, only contained *R. equi*. Therefore it seems that both individual species capable of producing the metabolite were required to be present in the mixtures studied in order for the metabolite to be produced and detected.

These results demonstrate that the ability of an individual bacteria to degrade a compound does not accurately represent what occurs by mixed bacterial cultures,thus a method to study the biodegradation of compounds using controlled mixtures of pure bacterial cultures has been developed.This provides an alternative to the traditional use of inocula from wastewater treatment plant (WWTP) activated sludge containing unknown and inconsistant microbial communities. The contradictory SMX removals observed at labscale (0 to 80%) are a result of this inconsistant nature of activated sludge indicating that the composition of a mixed bacterial culture strongly affects the results obtained. The method proposed in this study using a controlled mixture of pure bacterial cultures provides a laboratory technique to model the biological degradation of compounds that can be precisely repeated. This allows direct comparison of the results of experiments testing varying conditions which is not possible using activated sludge due to the unreliable nature of the microbial population.

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References

Al-Ahmad A, Daschner FD, Kümmerer K (1999) Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. Archives of Environmental Contamination and Toxicology 37 (2):158-163

Alexy R, Kumpel T, Kummerer K (2004) Assessment of degradation of 18 antibiotics in the Closed Bottle Test. Chemosphere 57 (6):505-512

Andersson DI (2003) Persistence of antibiotic resistant bacteria. Current Opinion in Microbiology 6 (5):452-456

Batt AL, Kim S, Aga DS (2007) Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. Chemosphere 68 (3):428-435

Benedict RG, Carlson DA (1971) Aerobic heterotrophic bacteria in activated sludge. Water Research 5 (11):1023-1030

Benotti MJ, Trenholm RA, Vanderford BJ, Holady JC, Stanford BD, Snyder SA (2009) Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. Environmental Science & Technology 43 (3):597-603. doi:doi:10.1021/es801845a

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

Carballa M, Omil F, Lema JM, Llompart M, Garcia-Jares C, Rodriguez I, Gomez M, Ternes T (2004) Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Research 38 (12):2918-2926

Cavallucci S (2007) Top 200: What's topping the charts in prescription drugs this year? Pharmacy Practice, Canadian Healthcare Network (http://www.imshealthcanada.com/vgn/images/portal/cit_40000873/13/31/8286270612-TOP200-07-final.pdf)

Costanzo SD, Murby J, Bates J (2005) Ecosystem response to antibiotics entering the aquatic environment. Marine Pollution Bulletin 51 (1-4):218-223

Daughton CG (ed) (2003) Chemicals from pharmaceuticals and personal care products, vol 1. Water: Sciences and Issues. MacMillan Reference, New York

Daughton CG, Ternes TA (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental Health Perspectives Supplements 107 (S6):907-938

Drillia P, Dokianakis SN, Fountoulakis MS, Kornaros M, Stamatelatou K, Lyberatos G (2005) On the occasional biodegradation of pharmaceuticals in the activated sludge process: the example of the antibiotic sulfamethoxazole. Journal of Hazardous Materials 122 (3):259-265

Gartiser S, Urich E, Alexy R, Kümmerer K (2007) Ultimate biodegradation and elimination of antibiotics in inherent tests. Chemosphere 67 (3):604-613

Göbel A, McArdell CS, Joss A, Siegrist H, Giger W (2007) Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Science of the Total Environment 372 (2-3):361-371

Goni-Urriza M, Capdepuy M, Arpin C, Raymond N, Caumette P, Quentin C (2000) Impact of an urban effluent on antibiotic resistance of riverine enterobacteriaceae and Aeromonas spp. Appl Environ Microbiol 66 (1):125-132. doi:10.1128/aem.66.1.125-132.2000

Hirsch R, Ternes T, Haberer K, Kratz K-L (1999) Occurrence of antibiotics in the aquatic environment. The Science of The Total Environment 225 (1-2):109-118

Janke D, Fritsche W (1985) Nature and significance of microbial cometabolism of xenobiotics. Journal of Basic Microbiology 25 (9):603-619

Jones O, Voulvoulis, N., Lester, J.N. (2005) Human pharmaceuticals in wastewater treatment processes, critical reviews. Environ Sci Technol 35 (4):401-427

Jorgensen SE, Halling-Sorensen B (2000) Drugs in the environment. Chemosphere 40 (7):691-699

Joss A, Keller E, Alder AC, Göbel A, McArdell CS, Ternes T, Siegrist H (2005) Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Research 39 (14):3139-3152

Joss A, Zabczynski S, Gobel A, Hoffmann B, Loffler D, McArdell CS, Ternes TA, Thomsen A, Siegrist H (2006) Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. Water Research 40 (8):1686-1696

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ Sci Technol 36 (6):1202-1211

Kümmerer K (2009a) Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75 (4):417-434

Kümmerer K (2009b) Antibiotics in the aquatic environment - A review - Part II. Chemosphere 75 (4):435-441

Kümmerer K (2009c) The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. Journal of Environmental Management 90 (8):2354-2366

Larkin MJ, Kulakov LA, Allen CCR (2005) Biodegradation and Rhodococcus - masters of catabolic versatility. Current Opinion in Biotechnology 16 (3):282-290. doi:10.1016/j.copbio.2005.04.007

Martínková L, Kren V, Cvak L, Ovesná M, Prepechalová I (2000) Hydrolysis of lysergamide to lysergic acid by Rhodococcus equi A4. Journal of Biotechnology 84 (1):63-66. doi:10.1016/s0168-1656(00)00332-1

Martínková L, Uhnáková B, Pátek M, Nesvera J, Kren V (2009) Biodegradation potential of the genus Rhodococcus. Environment International 35 (1):162-177. doi:10.1016/j.envint.2008.07.018

Miège C, Choubert JM, 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 (5):1721-1726

Peng X, Wang Z, Kuang W, Tan J, Li K (2006) A preliminary study on the occurrence and behavior of sulfonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China. Science of The Total Environment 371 (1-3):314-322

Perez S, Eichhorn P, Aga DS (2005) Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. Environmental Toxicology and Chemistry 24 (6):1361-1367

Rani A, Porwal S, Sharma R, Kapley A, Purohit HJ, Kalia VC (2008) Assessment of microbial diversity in effluent treatment plants by culture dependent and culture independent approaches. Bioresource Technology 99 (15):7098-7107

Reinthaler FF, Posch J, Feierl G, Wust G, Haas D, Ruckenbauer G, Mascher F, Marth E (2003) Antibiotic resistance of E. coli in sewage and sludge. Water Research 37 (8):1685-1690

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 (1):21-33

Ternes T, Janex-Habibi, M-L., Knacker, T., Kreuzinger, N., Siegrist, H, (2006a) 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/

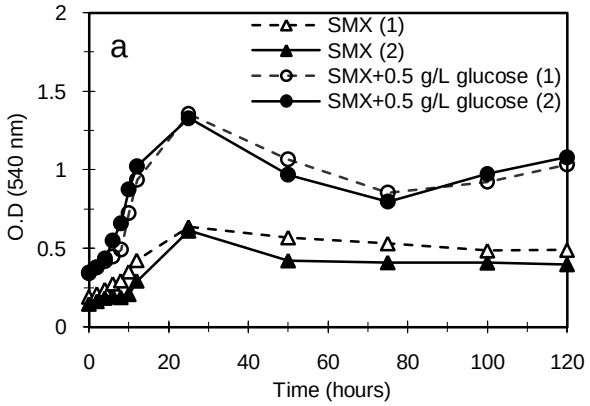
Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U (2004) Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). Journal of Microbiological Methods 56 (2):277-286

Xu W, Zhang G, Li X, Zou S, Li P, Hu Z, Li J (2007) Occurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China. Water Research 41 (19):4526-4534

Yargeau V, Lopata A, Metcalfe C (2007) Pharmaceuticals in the Yamaska River, Quebec, Canada. Water Quality Research Journal of Canada 42 (4)

List of Figures

Figure 1


a
SMX (1)
SMX (2)
SMX+0.5 g/L glucose (1)
SMX+0.5 g/L glucose (2)
O.D (540 nm)
Time (hours)
0
0.5
1
1.5
2
0
20
40
60
80
100
120

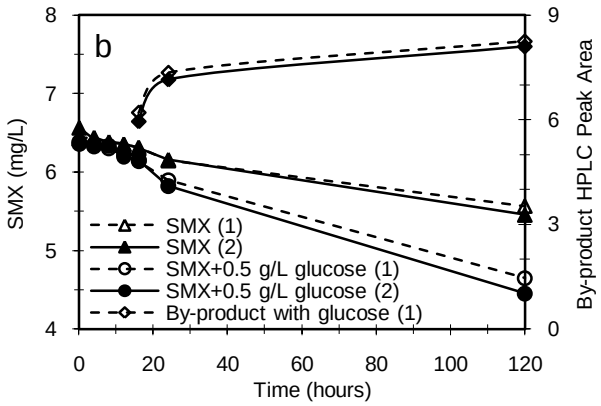
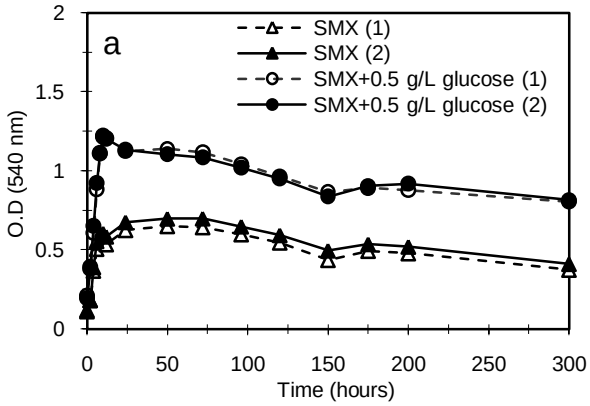
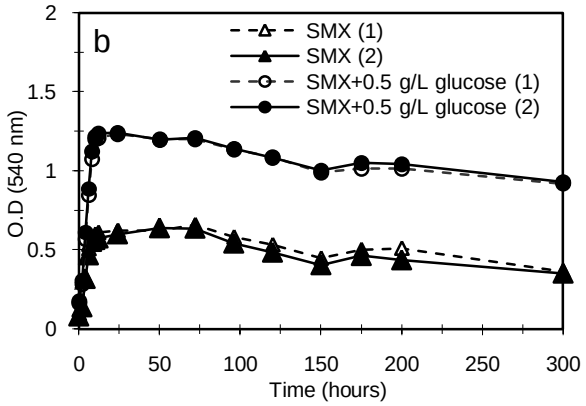
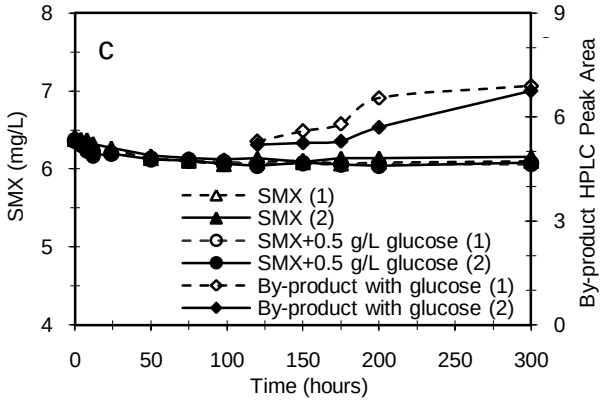

b
SMX (mg/L)
By-product HPLC Peak Area
Time (hours)
SMX (1)
SMX (2)
SMX+0.5 g/L glucose (1)
SMX+0.5 g/L glucose (2)
By-product with glucose (1)
8
7
6
5
4
9
6
3
0
0
20
40
60
80
100
120

Figure 2


a
SMX (1)
SMX (2)
SMX+0.5 g/L glucose (1)
SMX+0.5 g/L glucose (2)
O.D (540 nm)
Time (hours)
0
0.5
1
1.5
2
0
50
100
150
200
250
300


b
SMX (1)
SMX (2)
SMX+0.5 g/L glucose (1)
SMX+0.5 g/L glucose (2)
O.D (540 nm)
Time (hours)
0
0.5
1
1.5
2
0
50
100
150
200
250
300


C
SMX (mg/L)
By-product HPLC Peak Area
Time (hours)
SMX (1)
SMX (2)
SMX+0.5 g/L glucose (1)
SMX+0.5 g/L glucose (2)
By-product with glucose (1)
By-product with glucose (2)
8
7
6
5
4
9
6
3
0
0
50
100
150
200
250
300

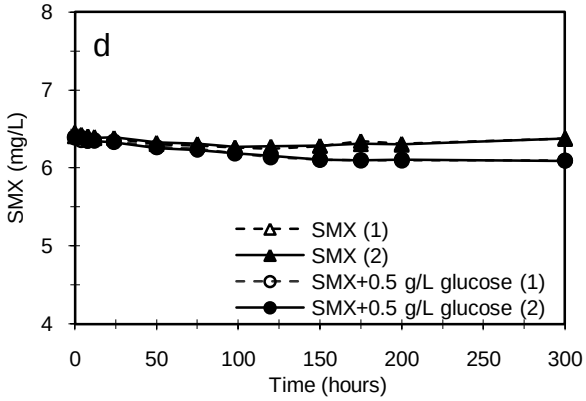

d
SMX (mg/L)
8
7
6
5
4
0
50
100
150
200
250
300
Time (hours)
SMX (1)
SMX (2)
SMX+0.5 g/L glucose (1)
SMX+0.5 g/L glucose (2)

Table 1 Summary of individual bacteria results: maximum growth (optical density measured at 540 nm) and SMX percent removal

Bacteria	Maximum Growth ($O.D_{540}$)[a]		SMX percent removal[b]		Detection of metabolite
	SMX	SMX + glucose	SMX	SMX + glucose	
Bacillus subtilis	0.28 0.24	0.43 0.40	< 1%	2.8% ± 0.03%	No
Pseudomonas aeruginosa	0.48 0.47	1.07 1.07	1.3% ± 0.15 %	5.6% ± 0.15%	Yes (with & without glucose)
Pseudomonas putida	0.46 0.46	1.25 1.25	0	0	No
Rhodococcus equi	0.64 0.61	1.36 1.33	15 % ± 1.6 %	29% ± 2%	Yes (with glucose)
Rhodococcus erythropolis	0.60 0.60	1.10 1.00	2.9% ± 0.15%	2.6% ± 0.12%	No
Rhodococcus rhodocrous	0.53 0.51	1.10 1.03	6.6% ± 0.9%	4% ± 0.2%	No
Rhodococcus zopfii	0.53 0.50	1.30 1.29	< 1%	< 1%	No

[a] maxium optical density values measured at 540 nm ($O.D_{540}$) for each duplicate experiment
[b] average SMX percent removal of duplicate experiments ± range in values (after 120 hours)