Phi Yen Nguyen
Mestre em Ciência Ambiental

Bioaugmentation for the removal of the antibiotic sulfamethoxazole in wastewater treatment plants

Dissertação para obtenção do Grau de Doutor em
Química Sustentável

Orientador: Maria d'Ascensão Miranda Reis, Professora Catedrática
Faculdade de Ciências e Technologia, Universidade Nova de Lisboa
Co-orientadores: Adrian Michael Oehmen, Professor Auxiliar,
Faculdade de Ciências e Technologia, Universidade Nova de Lisboa
Gilda Carvalho, Investigadora Principal,
Faculdade de Ciências e Technologia, Universidade Nova de Lisboa

Júri

Presidente: Prof. Doutora Ana Isabel Nobre Martins Aguiar de Oliveira Ricardo
Arguentes: Prof. Doutora Paula Maria Lima e Castro
Prof. Doutor Ricardo Manuel Nunes Salgado

Vogais: Prof. Doutora Maria d'Ascensão Miranda Reis
Prof. Doutora Olga Cristina Pastor Nunes
Doutora Nídia Dana Mariano Lourenço de Almeida

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

March, 2018

Bioaugmentation for the removal of the antiobiotic sulfamethoxazole in wastewater

AGRADECIMENTOS

During my Ph.D, I was able to work with many people from whom I have learned a lot, and many have also contributed greatly to my work. First and foremost, I would like to gratefully acknowledge my principal supervisor, Prof. Maria d'Ascensão Miranda Reis, and the two co-supervisors, Prof. Adrian Michael Oehmen and Dr. Gilda Carvalho, for offering me the opportunity to pursue my dream as well as for all their time and effort in instructing and guiding me during the course of my Ph.D. They provided me with the insightful comments, and solutions for my work. I greatly appreciate for your excellent support and encouragement, not only for my studies but also for many personal aspects.

I'd like to express my exceptional gratitude towards the other co-authours of our papers, Prof. Olga Cristina Pastor Nunes and Ana Reis from University of Porto, and Dr. Ana Filipa Silva from Instituto de Biologia Experimental e Tecnológica, for their significant contributions and expertise. Additionally, I also special thank to EPAL – Grupo Águas de Portugal for the collaboration in the project, especially to Eng. Maria João Araujo Gouveia Benoliel, Dr. Vitor Vale Cardoso, Alexandre Mourão Rodrigues, and Dr. João Eduardo Rodrigues, who offered significant support in the analytical methodology and laboratory. A big thank you also goes out to other co-supervisor, Prof. Benedek G. Plósz, and Dr. Elena and Dr. Fabio, for their significant contributions and many valuable comments to my work performed during my secondment at Technical University of Denmark. Also, thanks to Dr. Ricardo Salgado for his help with the analysis at the beginning of my PhD.

Thanks to Mermaid fellows who created my ever-lasting warm feelings and unforgettable memories of my Ph.D life. Also thanks to all my colleagues in BIOENG group for creating such a nice atmosphere at work. And thanks to my Vietnamese friends here in Portugal for your love-you really made my day.

Last, but not least, a big thanks to my family, especially to my mom Hoa, who had provided me with much love and encouragement throughout these years. A big pity that she could not be able to wait for my graduation but I believe that I am always blessed by her and she is always in my heart.

I would like to thank very much the excellent support of MERMAID, an Initial Training Network funded by the People Programme (Marie Skłodowska-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607492. The programme was responsible for funding my research, overseas conferences and schools, as well as my scholarship for the first three years. Thanks also to the additional fellowship that was provided to me by the UCIBIO (FCT/MEC UID/Multi/04378/2013, POCI-01-0145-FEDER-007728 for my last year. Thanks also go out to the Universidade Nova de Lisboa for provision of these scholarships. This piece of work would not exist without these useful inputs which I am truly grateful.

Above all I would like to thank the Lord Jesus Christ for all the great things and kind persons that you have provided to me.

Resumo

Os antibióticos são usados intensivamente na terapia médica, medicina veterinária e na indústria agrícola, resultando em emissões contínuas dos compostos em ETARs e recursos de água doce. Uma vez que as ETARs convencionais não são eficazes para remover completamente os antibióticos, aumentam também as preocupações relacionadas com a influência de seu modo de ação nas comunidades microbianas, bem como o risco para a saúde humana, através da promoção e disseminação de resistência aos antibióticos. O objetivo deste estudo é desenvolver uma estratégia eficaz e prática de bioaumento para a remoção do antibiótico sulfametoxazol (SMX) - um dos antibióticos sulfonamida sintéticos mais frequentemente detectados nas águas residuais. Esta tese revelou que uma estirpe identificada como *Achromobacter denitrificans* PR1, anteriormente isolada de lamas activadas (AS), é um organismo com potencial para bio-aumento da remoção de SMX em águas poluídas. A cinética de degradação de SMX deste organismo é estimulada na presença de substratos biogénicos, por exemplo, acetato/ácido succínico, sendo duas ou três ordens de grandeza maior do que a cinética de degradação de lamas activadas, em concentrações ambientalmente relevantes. O bio-aumento de AS com a estirpe PR1 (testes descontinuos) conduziu a taxas sessenta vezes superiores de biotransformação de SMX em comparação com AS, dentro de um ambiente complexo de fontes de carbono. Modelos de degradação biológica em ETAR foram calibrados para descrever com precisão o destino de sulfametoxazol e os dois metabolitos humanos deste composto, N_4-acetil-SMX e SMX-N_1-glicuronídeo, sob várias condições redox.

A estirpe foi posteriormente bio-aumentada em biorreatores de membrana operado sob condições aeróbias, que levou a uma melhor estabilização da remoção de SMX, especialmente em situações de cargas de choque de SMX. Alteração dos tempos de retenção hidráulicos e, portanto, da disponibilidade de substratos primários, afeta o cometabolism de SMX da estirpe bio-aumentada nas lamas ativadas. Após o bioaumento, observou-se a perda da viabilidade das estirpes introduzidas. Reinoculação da estirpe degradadora parece ser uma solução lógica para manter a eficiência de remoção do composto alvo.

Palavras-chave: farmacêuticos, cometabolismo, bio-aumento, modelação, biorreatore de membrana, águas residuais

Abstract

Antibiotics are intensively used in medical therapy, veterinary medicine, and the farming industry, resulting in continuous releases of the compounds into WWTPs and fresh water resources. Since conventional WWTPs are not effective to completely remove antibiotics, there are growing concerns related to the influence of its mode of action to microbial communities, as well as the risk to human health, by promoting and spreading antibiotic resistance. The aim of this research is to develop an effective and practical bio-augmentation strategy for the removal of the antibiotic sulfamethoxazole SMX - one of the most frequently detected synthetic sulfonamide antibiotics in wastewater. A strain identified as *Achromobacter denitrificans* PR1, previously isolated from activated sludge (AS), was found as a potential organism for bio-augmentation for SMX removal in polluted waters. The SMX degradation kinetics of this organism are stimulated in the presence of biogenic substrates, e.g. acetate/succinate, and are two to three orders of magnitude higher than the degradation kinetics of activated sludge at environmentally relevant concentrations. Bioaugmentation of AS with the strain PR1 (batch experiments) led to superior biotransformation rates of SMX (by sixty times) compared to AS, within a complex carbon environment in WWTPs. Biological degradation models were calibrated to describe accurately the fates of sulfamethoxazole and the two human metabolites, e.g. N_4-acetyl-SMX and SMX-N_1-Glucuronide, in the systems, under various redox conditions.

The strain was subsequently bioaugmented into membrane bioreactors operated under aerobic conditions, which led to the enhancement and stabilization of the SMX removal, especially when SMX shock loads occurred. Changing hydraulic retention times, and thus the availability of primary substrates, was found to affect the cometabolism of SMX by the bioaugmented strain in activated sludge. After the bioaugmentation, the loss of viability of the introduced strains was observed and re-inoculation of the degrading strain seems to be a logical solution to maintain removal efficiency of the target compound.

Keywords: pharmaceuticals, cometabolism, bioaugmentation, modelling, membrane bioreactor, human conjugates, retransformation

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOTATIONS AND ABBREVIATIONS

3A5MI	3-amino-5-methylisoxazole
ALR	Ammonium Loading Rate
AS	Activated Sludge
ASM	Activated Sludge Model
ASM-X	Activated Sludge Modelling framework for Xenobiotics
CAS	Conventional Activated Sludge
COD	Chemical Oxygen Demand
F/M	Food to Microorganism Ratio
HRT	Hydraulic Retention Time
MBR	Membrane Bioreactor
MP	Micropollutant
Ac-SMX	N_4-acetyl-sulfamethoxazole
OLR	Organic Loading Rate
qPCR	Real-time quantitative polymerase chain reaction
SMX	Sulfamethoxazole
SMX-Glu	Sulfamethoxazole-N_1-Glucuronide
SRT	Solids Retention Time
TSS	Total Suspended Solid
VSS	Volatile Suspended Solid
WWTP	Wastewater Treatment Plant
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistant Genes

SPECIFIC TERMS

C_{LI}	the aqueous concentrations of the parent compound
C_{CJ}	the aqueous concentrations of the human metabolites undergoing deconjugation to the parent compound
$k_{Dec,Ox}$	Aerobic biotransformation rate coefficient for the human metabolites, C_{CJ}
$k_{bio,Ox}$	Aerobic biotransformation rate coefficient for the parent compound, C_{LI}
$q_{bio,Ox}$	Aerobic cometabolic-biotransformation rate constant for parent compound, C_{LI}
$k_{Dec,Ax}$	Anoxic biotransformation rate coefficient for the human metabolites, C_{CJ}
$k_{bio,Ax}$	Anoxic biotransformation rate coefficient for the human metabolites, C_{LI}
S_S	primary substrate concentration (e.g., organic matter or acetate, expressed as readily soluble biodegradable COD) considering a primary substrate (gCOD L^{-1})
K_S	half-saturation coefficient for S_S
K_D	solid-water distribution coefficient
X_{PR1}	biomass concentration of bioaugmentation strain *A. denitrificans*, expressed in gCOD L^{-1}
X_{AS}	biomass concentration of activated sludge, expressed in gCOD L^{-1}
X_{SS}	suspended solids concentration
f_{OC}	organic carbon fraction
K_{OC}	organic carbon water partition coefficient
K_{OW}	octanol-water partition coefficient

1

INTRODUCTION

1.1. Micropollutants

The availability of a sustainable supply of fresh water has always been central to the development of urban centres around the world. Growing population and rising standards of living exert stress on water supply and on the quality of drinking water. Management of such issues involves multiple options including water recycling and wastewater reuse. However, the increasing worldwide contamination of freshwater systems with thousands of industrial and natural chemical compounds has become one of the big challenges for a sustainable water future. These chemicals are ubiquitously detectable in the environment at trace levels ranging from nanograms to micrograms per litre, and are often called micropollutants (MPs) (because their concentrations are very low), or emerging contaminants (since the concern about them is only recent) or xenobiotics (since most of them are synthetic) (Fatta-Kassinos et al., 2010). They are mostly synthetic substances such as pharmaceuticals, biocides, illicit drugs, cosmetics, pesticides, herbicides and surfactants, to name a few; and naturally occurring substances such as hormones. Within these categories, groups of chemicals with similar structures can be found. However, often groups of chemicals with very different structures belong to the same category.

MPs can enter in different environmental compartments by transport and distribution via several different routes and can end up in soil, ground water, or wastewater treatment plants (WWTPs) as a result of (i) domestic uses (e.g. of illicit drugs, pharmaceuticals, cosmetic and hygiene products), (ii) industrial discharge, (iii) agricultural uses (e.g. of antibiotics and pesticides/herbicides) (iv) stormwater runoff from cities. The application of pharmaceuticals in livestock, followed by fertilization with manure, could contribute to an additional source of pharmaceuticals in the aquatic environment (Boxall et al., 2004). Many pharmaceuticals are excreted from the human body in the form of unmodified parent compounds as well as conjugated forms which can enter the water cycle via wastewater.

These conjugates can be transformed back to the parent compounds, causing an increase or negative removal of the parent compound during wastewater treatment processes. Unfortunately, conventional WWTPs are not designed to remove or degrade MP residues, which then are discharged into the aquatic environment via treated water, or into terrestrial environment by disposal of the sewage sludge/biosolids in landfill. As a result, the constant re-introduction of MPs through discharge from WWTPs creates pseudo-persistence (i.e. continual and ongoing exposure) in the environment and aquatic organisms are permanently exposed to this pollution. Even at very low concentrations, MPs can exert considerable (eco)toxicological concerns (i.e. acute and chronic toxicity) on aquatic organisms and humans (Flaherty and Dodson, 2005; Hoeger et al., 2005; Lai et al., 2009; Murray et al., 2010). When chemicals with similar modes of action are present as complex mixtures, additive or synergistic effects can render such mixtures dangerously potent (Altenburger et al., 2004; Brian et al., 2005; Chèvre et al., 2006; Escher et al., 2011; Schwarzenbach et al., 2006). Endocrine disrupting chemicals can be linked to adverse health effects reported in humans and wild animals, for example, feminization (Jobling et al., 1998) and hermaphroditism in wildlife and the development of testicular and prostate cancer and decreased sperm reproduction in humans (Sonnenschein and Soto, 1998). Recently, the presence of antibiotics in the aquatic environment is a growing concern related to promoting of antibiotic resistant bacteria (Berendonk et al., 2015).

Within the European Union, although no legal discharge limits into the environment are currently defined, a list of prioritized substances that are seen as a threat to surface and ground water has been published. In the Water Framework Directive 2008/105/EU, environmental quality standards were defined for the list of 33 priority substances/groups to control the chemical status of waters. In 2013, the new Directive 2013/39/EU came into force with twelve additional substances introduced to the controlled lists and thus, in total, 45 compounds were classified as priority substances. Recently, the first watch list of 10 substances/groups of substances for European Union-wide monitoring was reported in the Decision 2015/495/EU of 20 March 2015. The watch list included the anti-inflammatory diclofenac and the synthetic hormone 17-alpha-ethinylestradiol-EE2, a natural hormone (17-beta-estradiol-E2), other natural hormone (estrone (E1), three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), an antioxidant (2,6-di-tert butyl-4-methylphenol) commonly used as food additive, a UV filter (2-ethylhexyl-4-methoxycinnamate), and some pesticides (methiocarb, oxadiazon, imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid and triallate) (Barbosa et al., 2016). However, there are more problematic substances than the ones defined at the EU level. For all substances not appearing on the EU lists, member states have to identify so-called water basin specific substances and define corresponding environmental quality norms. What remains fully unregulated is the question of which policy measures come into force if the quality norms are exceeded.

1.2. Fate of MPs in wastewater treatment plants

Conventional WWTPs can effectively remove solid wastes, particulates, easily biodegradable organic substances, nutrients (i.e. nitrogen and phosphorus), and pathogens from wastewater. Even though WWTPs have not been specifically designed for the removal of MPs, the compounds can be affected by physical, chemical, and biological processes occurring during the treatment. The behaviour of contaminants in WWTPs depends on the physical-chemical properties of specific compounds, their susceptibility to sorption, chemical degradation and biodegradability. The main mechanisms associated with MP removal in wastewater treatment are described as below:

1.2.1. Abiotic processes

A number of other processes can influence the removal of MPs in wastewater, e.g. photolysis, volatilization and stripping during aeration. Photolysis is a more relevant mechanism of MPs in stabilization ponds, or in ultraviolet disinfection. Due to the high turbidity of the wastewater and mixed sludge liquor, photolysis is considered as an insignificant process for the removal of MPs in activated sludge wastewater treatment (Michael et al., 2013). The extent of dissolved compounds being stripped from the water phase into the gas compartment depends on the physicochemical properties of the compounds (i.e. Henry's constant) and the aeration conditions. The effect is taken into account for a compound with a Henry coefficient > $3*10^{-3}$. Pharmaceuticals are mostly hydrophilic and have Henry's constant values < 10^{-5}, therefore the stripping efficiency is considered to be insignificant (<10%) (Ternes and Joss, 2006). Several studies demonstrated minor contributions of volatilization to the removal of pharmaceuticals in activated sludge processes (Göbel et al., 2005; Suárez et al., 2010).

1.2.2. Sorption/desorption

Sorption onto sludge can be an important removal mechanism of non-biodegradable MPs from WWTPs. In wastewater, MPs can sorb to solids as well as to dissolved and colloidal matter (Pomiès et al., 2013). It is composed of wo reactions: sorption from liquid to the solids and desorption from solids to the aqueous phase (Ternes and Joss, 2006). When the rate of the two respective processes are equal, sorption equilibrium is considered. Solid-liquid partitioning of a compound is characterized by using the sorption coefficient K_d (L gSS^{-1}). At equilibrium, the MP concentration sorbed onto sludge (C_S) is assumed to be proportional to the dissolved concentration (Joss et al., 2006a):

$$K_d = \frac{C_S}{C_{LI} X_{SS}} \tag{1.1}$$

where X_{SS} (gSS L^{-1}) is suspended solids concentration; C_{LI} (μg L^{-1}) is the dissolved concentration; C_S (μg L^{-1}) is the concentration sorbed onto sludge, per unit reactor volume.

And the total concentration of the compound C (μg L^{-1}) is defined as:

$$C = C_S + C_{LI} \tag{1.2}$$

In most cases, sorption and desorption rates are significantly faster than the hydraulic retention time or the MPs biotransformation rates, thus, it can be assumed to be in close equilibrium, if the sorption substance mass flux is significantly higher (about 10 times) than the biodegradation flux (Ternes and Joss, 2006). Sorption experiments in batch reactors also showed that equilibrium can be reached after 0.5-1h (Andersen et al., 2005; Hörsing et al., 2011; Ternes et al., 2004).

The extent of sorption can be affected by different factors such as the physico-chemical properties of the solids and the chemicals involved (Bowman et al., 2002) as well as the environmental conditions, i.e. pH, ion strength, temperature or the presence of complexing agents, (Spark and Swift, 2002). Insignificant sorption (< 10%) during wastewater treatment was observed for most of the investigated compounds (e.g. antibiotics, drugs, contrast media, fragrances, hormones) with K_d values < 0.3 L gSS^{-1} (Ternes and Joss, 2006). The process is mostly involved in two main mechanisms: (i) absorption – hydrophobic interactions of the aliphatic and aromatic groups of a compound with the lipophilic cell membrane of the microorganisms or the lipid fractions of the suspended solids; (ii) adsorption – electrostatic interactions between positively charged groups of chemicals with negatively charged polysaccharide structures on the outside of bacterial cells (Ternes and Joss, 2006).

The octanol-water partition coefficient (K_{OW}), which represents the hydrophobicity, is commonly used as a chemical-specific predictor of nonspecific sorption to organic matter (Hyland et al., 2012). Different sorption extent can be considered according to the K_{OW} : low sorption potential (log K_{OW}< 2.5), medium (2.5<log K_{OW}<4) and high sorption (log K_{OW}>4) (Rogers, 1996).

The sorption was also shown to be directly related to organic carbon content, represented by the organic-carbon water partition coefficient (K_{OC}) (Karickhoff et al., 1979), as below:

$$K_d = f_{OC} K_{OC} \tag{1.3}$$

where f_{OC} (g organic carbon $gTSS^{-1}$) denotes the organic carbon fraction in the solids.

Most of the time, to simplify, modellers considered K_d as a unique value represented for an intrinsic physical-chemical property of a MP (Pomiès et al., 2013). However, sorption extent depends also on the type of solid matrix (e.g., activated sludge, particulate content of raw/treated wastewater, soil, and sediment) and differentiation of K_d values are required. Indeed, sorption is not only associat-

ed with hydrophobic interactions but also with other mechanisms such as electrostatic interactions (Ternes et al., 2004), hydrogen bondings, cationic exchanges, cationic bridges, and surface complexation (Tolls, 2001).

Some MPs contain polar functional groups (e.g. carboxylic moieties, aldehydes and amines), which are ionized during wastewater treatment processes, i.e. anionic, cationic or zwitterionic, which can interact with special parts of organic matter or with minerals. Their partitioning behavior is affected by pH and ionic interactions and have been addressed in many studies. In these cases, the K_{OC} predictions were based on the octanol–water partitioning coefficient (Kow) for neutral compounds or the pH-dependent octanol-water distribution (D_{OW}) which considers the pKa at the ambient pH (Carballa et al., 2008; Drillia et al., 2005b; Radjenovic et al., 2009; Rosal et al., 2010).

1.2.3. Retransformation

Pharmaceuticals can be metabolized in the human body via two separate degradative processes: Phase I – introduction of polar group and Phase II – conjugation of polar group. The first reaction is the functionalization reaction, comprising hydrolysis (hydrolytic cleavages), reduction, and oxidation (alkylations and dealkylations), mostly catalyzed by cytochrome P-450 reductase and cytochrome P-450 V (Roth, 1997). Phase II includes glucuronidation (transfer of glucuronic acid to phenols, aliphatic hydroxyl, carboxyl, thiol, amine and hydroxylamino groups), along with less frequent sulfation, glutathione conjugation, N-acetylatioin and amino acid conjugation etc. (Roth, 1997). Once administered, pharmaceuticals are metabolized and excreted in the form of unchanged compounds along with considerable amounts of human conjugated forms (may be up to 22% of the excreted pharmaceuticals (Testa et al., 2012)). There is a very limited knowledge on the environmental fate and behavior of those human metabolites. 'Negative removal' of MPs has previously been described in WWTPs (Stadler et al., 2012) when comparing initial and final concentration, and likely explained as a result of different processes, e.g. retransformation or deconjugation of human conjugates (metabolized or conjugated forms of parent pharmaceuticals) back to parent compounds (Polesel et al., 2016) etc.. Several lines of evidence show that pharmaceutical metabolites are cleaved back to the parent compounds in the sewer or in WWTPs by widely available biological reactions or even by abiotic processes for several pharmaceuticals, e.g. sulfamethoxazole (Göbel et al., 2007; Joss et al., 2006b; Stadler et al., 2015), sulfapyridine (García-Galán et al., 2012), diclofenac and carbamazepine (Plósz et al., 2012) etc.. Notably, for many pharmaceuticals, conjugated or metabolized forms are present at even higher concentration, e.g. N_4-acetyl-SMX (Ashton et al., 2004; Göbel et al., 2005), SMX-Glu (Wang and Gardinali, 2014) than the parent compound in WWTP effluents (Stadler et al., 2012). Thus, attention must be paid to the conjugated forms of pharmaceuticals and their fates in WWTPs and in the environment to gain a more comprehensive understanding about their removal during wastewater treat-

ment, avoiding discharge of compounds that could retransform back into their active parent forms in the environment (Stadler et al., 2015).

A number of other processes are also responsible for 'negative' removal efficiencies observed in WWTPs, such as abiotic retransformation of metabolites and transformation products; formation from analogues and structurally related chemicals; or releases from faecal matter and hydrolysis of particulate and colloidal matter, or even desorption (Polesel et al., 2016).

1.2.4. Biotransformation/biodegradation

Biotransformation/biodegradation are typically referring to biologically-mediated/enzyme catalysed chemical conversions that results in (i) the formation of other transformation products (metabolites) with a slightly modified structure; or (ii) either the loss of certain chemical properties (primary biodegradation) or fully reduced or oxidized products such as carbon dioxide and water (ultimate biodegradation) (Kolvenbach et al., 2014).

The conversion of MPs sometimes could lead to formation of intermediates that may be more biodegradable and would enter the central metabolic pathways for further biotransformation (Yi and Harper, 2007). Transformation products and intermediary degradation products can also be similar/more persistent and toxic compared to the parent compounds and can / cannot be further assimilated by other microbes present in the mixed activated sludge (Khunjar et al., 2011; Liu et al., 2013; Quintana et al., 2005). Thus, there is a need to include identifications of transformation products in environmental risk assessments.

In a mixed culture such as activated sludge, removal of the MPs in general, especially polar ones, could be realized mainly via biodegradation, either due to metabolic (e.g. heterotrophic microbes) or co-metabolic activities of activated sludge degraders, e.g. heterotrophic and autotrophic microorganisms (Tran et al., 2013). To date, biotransformations carried out by specific degraders that could use MPs as sources of carbon and energy to maintain cell growth are still limited. Heterotrophic microbes in the environment are known to be able to degrade a large variety of MPs via their various monooxygenases and/or dioxygenases (Arp et al., 2001; Khunjar et al., 2011). Metabolic degradation of MPs by pure culture or mixed activated sludge has been reported for several compounds such as endocrine disrupters (Lindblom et al., 2009), the antibiotic sulfamethoxazole (Larcher and Yargeau, 2011; Reis et al., 2014); ibuprofen (Murdoch and Hay, 2005); estradiol (Iasur-Kruh et al., 2011); acetaminophen (De Gusseme et al., 2011) etc.. Metabolic degradation of xenobiotics often results in low net growth of biomass and slow degradation rate, thus, the presence of a biogenic substrate (a readily biodegradable substrate) was demonstrated to not only increase biomass but also decrease lag-phase

and augment the degradation kinetics of other xenobiotic compounds (Chong et al., 2012; Oehmen et al., 2013).

Since MPs are detected in the environments at concentrations (in the range of ng L^{-1} to μg L^{-1}) too low to be utilized for biomass growth and cell energy requirements as well as inducing the relevant enzymes for/cofactors involved in the biodegradation, cometabolism is believed to be the dominating biodegradation process. Cometabolism is defined as the biological transformation of a non-growth (co-metabolic) substrate by bacteria in the obligate presence of a primary substrate (or growth substrate) or another transformable compound (Dalton and Stirling, 1982). The roles of primary substrate in this case are to induce enzyme production, e.g. non-specific enzymes, for the degradation of MPs as well as provide energy for cell growth and maintenance. A number of studies have demonstrated that the biotransformation of some pharmaceuticals are likely to be due to co-metabolism (Gauthier et al., 2010; Müller et al., 2013; Plósz et al., 2012; Su et al., 2015).

Autotrophs also play important roles in co-metabolism of MPs via non-specific enzymes. Ammonia oxidizing bacteria (AOB) are known to possess ammonia monooxygenase (AMO), an enzyme with relatively broad substrate specificity that catalyzes nonspecific oxidation of many MPs (Khunjar et al., 2011). Several studies have demonstrated significant associations between nitrification activities of nitrifying activated sludge and elimination of many MPs such as pharmaceuticals, pesticides, and estrogens (Dytczak et al., 2008; Fernandez-Fontaina et al., 2012; Damian E Helbling et al., 2012; Shi et al., 2004; Yi and Harper, 2007).

Consequently, no matter how biotransformations of MPs happen via metabolic or co-metabolic processes, the presence of readily biodegradable substrates as co-substrates or biogenic substrates play a significant role in supporting the induction of catabolic enzymes (specific or non-specific), supplying energy and building blocks for the synthesis of proteins and biomass growth, etc.

Nevertheless, diauxic or sequential degradation, termed as competitive inhibition, when mixed substrates are present, has been observed between the growth- and co-metabolic substrate as a result of competition for non-specific enzymes (Joss et al., 2004; Li et al., 2008; Plósz et al., 2010b; Sathyamoorthy et al., 2013). Subsequently, biotransformation rates of co-metabolic substrates may be lower and vice versa (Chang and Alvarez-Cohen, 1995).

Modelling is considered a useful tool to understand the fate of MPs and their eliminations through WWTPs, which allows process optimization to reduce emissions of MPs in the treated effluent as well as represent support operators and legislators in making decisions. A comprehensive summary on current status of modelling of MPs' fate and transport in wastewater could be found in (Clouzot et al., 2013) and (Plósz et al., 2013a). In terms of modelling, biotransformation of organic chemicals generally can be written as a simplified version of the well-known Monod-model. However,

MPs and human metabolites have been found at low concentrations in wastewater, from ng L^{-1} to μg L^{-1}, which is significantly lower than the half-saturation coefficient. Thus the biomass transformation capacity increases linearly with the soluble concentration of the MP, C (μg L^{-1}), and could be described with the pseudo first-order kinetic expression (Joss et al., 2006b):

$$\frac{dC}{dt} = -k_{bio} X_{SS} C \tag{1.4}$$

where k_{bio} (L gX_{SS}^{-1} d^{-1}) is the reaction rate coefficient and X_{SS} is the suspended solids concentration inside the reactor (kg TSS L^{-1}). 'Pseudo' refers to the dependency from the term X_{SS}, assumed constant for short-term batch experiments (Joss et al., 2006b). And when sorption equilibrium is assumed Eq. 1.4 can be written:

$$\frac{dC}{dt} = -\frac{k_{bio}}{1 + K_D X_{SS}} X_{SS} C \tag{1.5}$$

where K_D is the solid-water distribution coefficient of the compound (L kg^{-1}).

Considering that MPs are present in concentrations significantly lower than their affinity constant, those are probably too low to induce the catabolic genes (Kolvenbach et al., 2014), and thus biotransformation may be dependent on co-metabolism catalysed by non-specific enzymes, in the presence of another carbon source available at higher concentration defined as the growth substrate (or primary substrate). The kinetics of co-metabolism was first developed by Criddle (1993), known later as reductant model by (Alvarez-Cohen and Speitel, 2001; Liu et al., 2014), and considered the beneficial effect of growth substrate availability on xenobiotic trace chemical biotransformation.

The Activated Sludge Modelling framework for Xenobiotics (ASM-X) model, an extension of the well-known Activated Sludge Models (ASMs) (Henze et al., 1999, 1987), was first identified and calibrated for antibiotics (Plósz et al., 2010b) during WWTPs. Then it was also extended to successfully calibrate and predict the fate of several other xenobiotic chemicals, such as diclofenac and carbamazepine (Plósz et al., 2012); cocaine (Plósz et al., 2013b); SMX, tetacycline and ciprofloxacin in full-scale WWTP (Polesel et al., 2016); illicit drug biomarkers (Ramin et al., 2016) in sewer systems; as well as a broad range of micropollutants in biofilm systems (Polesel et al., 2017; Torresi et al., 2017). In general, the cometabolic biotransformation model of MPs used in this study developed for mixed activated sludge culture by (Plósz et al., 2012) is characterized by two-rate process: the enhanced rate in the presence and the pseudo-first order rate in the absence of growth substrates, S_S, respectively:

$$\frac{dC}{dt} = -(q_{bio} \frac{S_S}{S_S + K_S} + k_{bio}) X_{SS} C \tag{1.6}$$

where q_{bio} (L gX_{SS}^{-1} d^{-1}) defines the cometabolic-biotransformation rate constant in the presence of the primary substrate (S_S) and K_S (g L^{-1}) is the primary substrate half saturation coefficient.

In case deconjugation of human metabolites is taken into account, a retransformation-biotransformation model describes the variation of aqueous concentration of the parent compounds (C_L) and a term is added to Eq. 1.7 as follows:

$$\frac{dC}{dt} = -(q_{bio}\frac{S_S}{S+K_S} + k_{bio})\frac{X_{SS}C_L}{1+K_D X_{SS}} + k_{dec}C_{CJ}X_{SS} \quad (1.7)$$

where C_{CJ} (g L^{-1}) accounts for the concentration of substances, biotransformed via the parent compound; C_L (g L^{-1}) is the dissolved parent compound; and k_{dec} (L g^{-1} d^{-1}) defines the retransformation rate constant. Negligible sorption can be considered for the fraction C_{CJ} due to its higher water-solubility than parent substances to improve their excretion from the human body (Plósz et al., 2010b).

1.3. Environmental factors affecting the removal of MPs

The removal of MPs in WWTPs is affected by many factors including compound physico-chemical properties; prevailing environmental factors such as redox conditions; pH; the presence of organic matter; or operational parameters such as hydraulic retention time (HRT), solid retention time (SRT) and environmental temperature.

1.3.1. Compound structures

Chemical structure of MPs provides not only information related to classification grouping, but also degradability or persistence of the compounds in the environment. Some studies have demonstrated that the chemical structure and physicochemical properties are important factors in evaluating the removal potential of MPs from WWTPs (Cirja et al., 2008; Kimura et al., 2005; Tadkaew et al., 2011). Bertelkamp et al., (2016) reported a statistically significant relationship between MP biodegradation rates and the functional groups of the molecular structures. The authors found that MP biodegradation rates increased in the presence of carboxylic acids, hydroxyl groups, and carbonyl groups, but decreased in the presence of ethers, halogens, aliphatic ethers, methyl groups and ring structures in the chemical structure of the MPs. An example from other studies of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), where the difference in structure of the two compounds is only the ethinyl group present in EE2, which makes it very recalcitrant, while the removal of E2 occurs quite easily during wastewater treatment (Ternes et al., 1999). Complex structure compounds with two aromatic rings, e.g. ketoprofen, mefenamic acid and naproxen, are presumably not efficiently removed by conventional activated sludge (CAS), but could be well removed by membrane bioreactor (MBR) supposedly due to their long SRT (Kimura et al., 2005). The authors also demonstrated that harbouring chlorine group compounds, e.g. diclofenac, dichloprop and clofibric acid, are often poorly removed by

both conventional activated sludge (CAS) and membrane bioreactor. This is in agreement with (Andreozzi et al., 2006), who observed that the presence of a nitro- group or a chlorine atom in an aromatic ring results in a decreased biodegradation rate attributed to the electron-withdrawing character of these substituents and of the electrophilic nature of the oxygen transfer to the reacting molecules. Tadkaew et al., (2011) found a correlation between molecular structures and the removal of MPs and proposed a qualitative framework for the prediction of trace organic removal by MBR treatment. The authors suggested that very hydrophobic MPs have log D > 3.2 (log D: pH-dependent distribution coefficient) and were grouped into very high removal compounds (ranging from 85% to more than 98%). Low removal efficiency (< 20%) was observed for hydrophilic and moderately hydrophobic (log D < 3.2), where compounds possessed strong electron withdrawing functional groups. In contrast, compounds bearing only electron donating functional groups such as hydroxyl groups and amine groups were reported for high removal (>70%). However, there were some exceptions which remained unexplainable due to the lack of biochemical data. During biodegradation, depending on the chemical structures of MPs, e.g., the presence of secondary, tertiary or quaternary carbon atoms as well as specific functional groups (Zhang et al., 2014), MPs may be mineralized or transformed to either more hydrophobic or more hydrophilic derivatives (Halling-Sørensen et al., 1998; Muter et al., 2017; Zhang et al., 2014).

1.3.2. Operational conditions

Biological wastewater treatment, e.g. the activated sludge process, has been noted to contribute significantly to removal of many MPs (Joss et al., 2005; Watkinson et al., 2007) and operational parameters such as hydraulic retention time (HRT), sludge retention time (SRT), pH and temperature etc., might influence and cause disparity in removal efficiencies for similar technologies reported in literature (Drewes, 2007).

Several studies stressed the significant impact of sludge retention time (SRT) on MP removal (Clara et al., 2005a; Petrie et al., 2014; Stasinakis et al., 2010; Suárez et al., 2012). High SRTs facilitate the enrichment of slowly growing bacteria and consequently, the establishment of a more diverse biocoenosis with broader physiological capabilities compared to WWTPs operating at low SRTs (Clara et al., 2005a). It is known that if removal of specific MPs is dependent on the SRT, and critical SRTs must be met, e.g. 10 days (Clara et al., 2005a) or 20 days (Plósz et al., 2012) for diclofenac, for their complete degradation in WWTPs. An activated sludge system operated at prolonged SRTs generally correlates with enhanced removal of some MPs (Göbel et al., 2007; Kimura et al., 2007; Kovalova et al., 2012). Clara et al., (2005) found higher removal rates at SRTs higher than 10 days for some compounds such as 17β-estradiol, estrone, estriole, ibuprofen, bezafibrate, and bisphenol-A. In contrast, in other studies, the highest biotransformation rates of endocrine disruptors was observed for

continuous AS systems operated at low SRT of 3 days compared to 10 and 20 days (Stasinakis et al., 2010). Majewsky et al., (2011) demonstrated that attenuation of intermediate biodegradable substrates, such as diclofenac and for SMX, is expected to be decreased at higher SRT due to a lower active biomass presence. In addition, several studies found little or no effect of increased SRT on the biotransformation of some MPs, e.g. carbamazepine (Clara et al., 2005a; Plósz et al., 2012), 17α-ethinylestradiol (Gaulke et al., 2009), paracetamol and caffeine (Majewsky et al., 2011) etc. In summary, the impact of SRT on biotransformation of MPs depends much on the chemical compounds to be degraded and no generalization on the impact of SRT could be postulated for all MPs.

The hydraulic residence time (HRT) also impacts the removal of pollutants. Systems with longer HRT appear to have better removal of MPs (Gros et al. 2010; Petrie et al. 2014). Higher removal of estrogen with longer HRT was attributed to a decrease in food to microorganism (F:M) ratio that led to biodegradation of less-favoured carbon substrates as well as increased contact time for biodegradation (Petrie et al., 2014). The long contact time between biomass and aqueous concentration of MP can be beneficial for the general slow removal rates estimated for MPs.

Temperature can affect microbial growth and activity. Several studies have focused on the effect of temperature on the biodegradation of MPs (Göbel et al., 2007; Hai et al., 2011; Suárez et al., 2005), but no direct relationship has been reported so far.

pH variations can affect the speciation of the ionizable compounds and subsequently their tendency of sorption to the sludge particles or bioavailability for biodegradation. For example, increases in removal efficiency of certain acidic MPs such as ketoprofen, diclofenac, ibuprofen, and sulfamethoxazole has been demonstrated when MBRs were operated under acidic conditions compared to neutral conditions. The reason was attributed to the speciation of the compounds from hydrophilic ionic forms to much more hydrophobic forms at pH lower than their acid dissociation constant (pKa) making the compounds more readily available for biomass sorption (Tadkaew et al., 2010). In contrast, no changes in the removal rate of non-ionizable compounds were expected when pH varied. Overall, pH of an aqueous environment can affect the physiology of microbes and also solubility, ionic and/or non-ionic states of MPs that subsequently influence MP eliminations (Tran et al., 2013).

1.3.3. Redox conditions

Removal of MP has been extensively studied under different redo conditions in wastewater treatment, e.g. aerobic, anoxic and anaerobic conditions (Alvarino et al., 2016; Falas et al., 2016; Joss et al., 2004; Stadler et al., 2015; Suárez et al., 2010). Previous studies have shown that the availability of electron acceptors, e.g. oxygen, nitrate or others, could influence the transformation rate of MPs. In general, it has been observed that degradation rates for MPs under aerobic conditions was faster than anaerobic and anoxic rates (Joss et al., 2004; Suárez et al., 2010). For instance, faster degradation

rates were observed for the majority of the 16 investigated pharmaceutical and personal care products in a nitrifying reactor compared to a denitrifying reactor (Suárez et al., 2010). Joss et al., (2004) observed significantly higher degradation rates of estrone E1 and estradiol E2 under aerobic conditions compared to anaerobic conditions. Anaerobic processes for MP removal were characterized as compound-specific (Muter et al., 2017).

In contrast, some chemicals had similar (e.g., N_4-acetyl-sulfamethoxazole, atenolol, clarithromycin) or higher (i.e., levetiracetam) removal rates under anoxic conditions than under aerobic conditions (Falås et al., 2013). There was also a few notable exceptions (Alvarino et al., 2016) such as decoloration of azo dyes (van der Zee and Villaverde, 2005) or deionisation of diatrizoate, and demethylation of venlafaxine and its metabolite O-desmethylvenlafaxine (Falas et al., 2016) were found to persist under aerobic conditions and only degraded under anaerobic conditions. In addition, redox gradients developed within a biofilm may induce highly stratified microbial communities, which was considered as one of the reasons why considerably higher removal of some pharmaceuticals, e.g. diclofenac, ketoprofen, gemfibrozil, and clofibric acid, in a moving bed biofilm reactor compared to activated sludge (Falås et al., 2012). Thus, it should be noted that different redox conditions impact the removal of different compounds. Furthermore, combinations of different redox potentials, i.e. aerobic, anoxic and anaerobic conditions, for wastewater treatment could broaden the spectrum of miropollutants susceptible to biological degradation, with restriction to some of the MPs that were found to be persistent in all biological treatments (Falas et al., 2016).

1.3.4. Presence of primary substrate

As mentioned above, primary growth substrates, e.g. organic carbon or ammonia, play an important role in the biological removal of MPs by inducing enzymes or supplying energy flow for biomass growth and maintenance, etc. However, the presence of the main substrates for microbial growth has been reported to exert multiple interactions on the removal of MPs such as enhancement (Oehmen et al., 2013; Plósz et al., 2012; Su et al., 2015; Tran et al., 2013, 2009); or inhibition (Plósz et al., 2010b; Sathyamoorthy et al., 2013) as a result of competition for non-specific enzyme active sites (Criddle, 1993). The composition and concentration of growth substrates have been shown to act as microbial selectors that could affect the structure and performance of the microbial community and gene expressions, which may in turn alter the MP degradation via changes in the individual populations present over an extended period of time (Alidina et al., 2014; Li et al., 2014). Other studies found that biotransformation of different compounds respond differently to the presence of readily degradable carbon in short term tests (Su et al., 2015; Tan et al., 2013). Tan et al., (2013) observed enhanced estrogen E1 degradation under prolonged biomass starvation conditions over 5-13 days, which was attributed to stimulation of multiple substrate utilizing degraders under low substrate conditions. Un-

der oligotrophic conditions, bacterial cells can develop a "multivorous" strategy to simultaneously metabolize dozens of different carbon substrates (metabolic flexibility) instead of specialization on a particular substrate and strict metabolic control via mechanisms such as catabolite repression (Egli, 2010). Improved 17α-ethinylestradiol (EE2) kinetics in activated sludge treatment was further reported as a result of population selection with growth at low organic substrate concentrations (Ziels et al., 2014).

Moreover, biotransformation of specific MPs has been proved to be correlated to nitrification (Fernandez-Fontaina et al., 2012; Damian E Helbling et al., 2012; Kassotaki et al., 2016; McAdam et al., 2010; Roh et al., 2009; Yi and Harper, 2007). As referred previously, cometabolism of MPs by ammonia oxidizing bacteria (AOB) has been reported via the broad spectrum non-specific enzyme of ammonia monooxygenase (AMO) (Batt et al., 2006b; Khunjar et al., 2011; Shi et al., 2004; Tran et al., 2009), which could also be suppressed under high concentrations of ammonia (Fernandez-Fontaina et al., 2012). In contrast, some studies tested the use of AMO inhibitors, which showed little to no effect on the biotransformation of MPs (Gaulke et al., 2008; Khunjar et al., 2011), suggesting that other enzymes, e.g. hydroxylamine oxidoreductase or nitrite oxidoreductase that are involved in the ammonia oxidation process, could be potentially responsible for the MP biotransformation (Damian E. Helbling et al., 2012). Thus, the relative contribution of AMO to biotransformation of MPs compared to enzyme from heterotrophs still remains unclear.

1.4. Bioaugmentation for MP removal from WWTPs

The main removal mechanisms for MPs in activated sludge systems in conventional WWTPs are biodegradation and abiotic degradation, including sorption, stripping and volatilization. Advanced or tertiary treatments integrated in some WWTPs, e.g. membrane filtration, activated carbon adsorption (Li and Zhang, 2011; Pocostales et al., 2010), or advanced oxidation with ozone and chlorine (Knopp et al., 2016; Pereira et al., 2007) etc., are the most effective to enhance the removal of MPs in the effluent of WWTPs, but often employed high operational and maintenance costs. In addition, ozonation and chlorine treatment have been shown to form persistent oxidation products which are equal or more toxic than the parent chemicals (Fatta-Kassinos et al., 2011), underpinning the need to optimize biological wastewater treatment. Biological treatments are considered as cost-effective and sustainable abatement of MPs. Unfortunately, conventional biological treatments are not efficient to treat wastewater that receives a high diversity of these emerging pollutants, as many of these are xenobiotics. It is commonly thought that the inefficiency is caused by a lack of necessary catabolic genes in the indigenous microbial community (Yao et al., 2013). A novel approach that allows for a better removal of MPs in wastewater is bioaugmentation, i.e. the addition of indigenous or allochthonous (non-indigenous) specialized microbial strains/microbial consortia or genetically modified organisms to pol-

luted hazardous waste sites or bioreactors in order to enable or enhance the biodegradation of targeted pollutants (Boon and Verstraete, 2010; Chen et al., 2012; Ma et al., 2009; Tian et al., 2006). Recently, bioaugmentation of phenol degrading microorganisms has been successfully applied in a field study using a bioreactor (400 m^3) for the biological treatment of phenol in industrial wastewaters (Poi et al., 2017). Complete phenol degradation was achieved, enabling a cost saving of US$30 per tonne compared to US$100 with conventional technologies. This case study suggested that bioaugmentation represents a promising, sustainable and cost-effective approach for the degradation of xenobiotics in wastewater.

Bioaugmentation for remediation of contaminated soils has been effectively recognized since 1970s (Ellis et al., 2000; Han et al., 2000; Major et al., 2002; Yao et al., 2013), and intensively studied to enhance removal of chlorinated pollutants (Frascari et al., 2010; Santharam et al., 2011) in wastewater; phenolic compounds (Duque et al., 2011; El-Naas et al., 2009; Fang et al., 2013) in wastewater; wastewater containing 3-chloroaniline (Boon et al., 2002); and other recalcitrant pollutants (Fang et al., 2013; Li et al., 2013; Ma et al., 2009; Wang et al., 2014; Wen et al., 2013) etc.. These examples serve as a proof-of-principle of the efficacy of bioaugmentation to remove recalcitrant /xenobiotic chemicals from wastewater.

Nevertheless, studies on bioaugmenation for MPs removal have been very limited. Roh and Chu, (2011) investigated performance of 17 β-estradiol (1 mg L^{-1}) removal in a lab-scale nitrifying activated sludge SBRs bioaugmented with a Sphingomonas strain KC8 at different SRTs, i.e. 5, 10 and 20 days. The results showed that long SRTs (i.e. 10 d and 20 d) were needed for the removal of estrogen to no estrogenic activity endpoint. Even though high removal of E2 (>99%) was observed in all reactors, the effects of bioaugmentation of strain KC8 would not be fully assessed due to the presence of unknown estrogen-degraders in the activated sludge background (no non-bioaugmented control reactors were conducted in concomitance for comparison). Iasur-Kruh et al., (2011) was successful in integration of E2-degrading bacteria, EDB-LI1, into a wetland pond biofilm to enhance removal of estradiol. Another example, Hashimoto et al., (2010) also succeeded in bioaugmentation of an estradiol-degrading bacteria, i.e. *Novosphingobium* sp. strain JEM-1, into a bench-scale conventional activated sludge system. Successful bioaugmentation of MP removal is considered when multi-criteria are met, e.g. the bioaugmented strains can grow and remove the MPs to levels below typical WWTP effluent levels within a complexed-substrate background as wastewater at practical degradation rates (Zhou et al., 2013). Fenu et al., (2015) introduced a *Microbacterium* sp. strain BR1 in a pilot scale MBR treating full-scale MBR effluent (post treatment) and treating raw municipal wastewater. Improvement in SMX removal was not observed in the two MBRs, except for the test with SMX concentrations far higher than the municipal wastewater relevant values. The failure in SMX removal upon bioaugmentation was due to the fact that the municipal MBRs were operated at low SRT compared to

the doubling time of *Microbacterium* sp., making no chance for the survival of the strain in realistic application.

The main challenges in achieving successful bioaugmentation is the survival/stability and incorporation of the introduced microorganism into the biological treatment system (Yao et al., 2013). The failed bioaugmentations (Bouchez et al., 2009; Goldstein et al., 1985) were attributed to various possible explanations (Boon and Verstraete, 2010): (i) the contaminant concentrations may be too low for maximal induction of or recognition by the catalytic enzymes (Horemans et al. 2013); (ii) the presence of inhibitors such as antibiotics, antiseptics etc. (iii) the growth rate of the augmented organism may be slower than the rate of removal due to the loss of degrading microorganisms, washed out of the system or not successfully competing with the indigenous bacterial community or grazed by protozoa (Bouchez et al., 2000); (iv) the other key substrates may be required for the inoculum; (v) the organism may physically fail to reach the pollutant or (vi) problems related to the adaptation of the inoculated microorganisms to the new environment (Qu et al., 2006).

In addition, the identification and isolation of appropriate microbial strains used in bioaugmentation are also one of challenges to success. Some bacteria are able to flocculate naturally or do develop hydrophobic cell walls which may enable their penetration and adhesion to flocs (Bouchez et al., 2009). The selected strains have to meet at least three criteria, i.e. active, persistent and compatible, as described by (Yu and Mohn, 2002). The biodegradation in a consortium-augmented bioreactor was found to be more effective compared to the isolated one (Yao et al., 2013). It was supposed that not all the strains in the consortium are specialized-degraders, but the non-specialized microorganisms in the consortium can help to successfully compete with indigenous microorganisms; or further utilize the degradation intermediates generated by other strains (Mrozik and Piotrowska-Seget, 2010).

In general, though bioaugmentation is powerful in wastewater treatment, successful bioaugmentation remains controversial. Very little is known about the removal dynamics of microbial communities in augmented systems (Qu et al., 2006). The key part of the process is activated sludge, but it is still considered as an impenetrable "black box" (Dabert et al., 2002). However, by the recent advances made in ecological studies of microbial communities, microbiology, molecular biology and bioengineering will provide useful information to manipulate the cells in different ways to increase the survival and metabolic rates of the inoculated cells, improving the design and performance of treatment systems (Boon and Verstraete, 2010; Dabert et al., 2002; Wagner et al., 2002).

1.5. Objectives and outline of this research

Amongst pharmaceuticals, antibiotics have been intensively used in human therapy for infectious diseases, veterinary medicine, livestock as growth promoters, in agriculture and aquaculture, re-

sulting in release detection of large amounts of antibiotics in the environment and water resources (Gothwal and Shashidhar, 2015; Li, 2014; Luo et al., 2014; Verlicchi et al., 2015). The discharge of antibiotics into the environment has become a growing concern because of, not only the influence in its mode of action to microbial communities (Fent et al., 2006), but also the risk to human health by promoting antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Berendonk et al., 2015). ARG or ARB with resistance to, e.g. ciprofloxacin, sulfamethoxazole, trimethoprim, quinolone, vancomycin etc, have been detected in different environments (Berendonk et al., 2015; Leonard et al., 2015; Martínez, 2008), and has become a critical global public health issue of this century (WHO 2014).

The focus of this PhD is on the removal of the antibiotic sulfamethoxazole (SMX, $C_{10}H_{11}N_3O_3S$), one of the most widely used synthetic sulfonamide antibiotics worldwide (Akhtar et al., 2011; Kumar and Xagoraraki, 2010). After oral administration and body metabolism, approx. 45-70% of a SMX dose is excreted from the human body (Radke et al., 2009) as the unchanged compound (15%-25%) as well as the conjugated forms N_4-acetyl-sulfamethoxazole (>40%) and sulfamethoxazole-N_1-glucuronide conjugate (9-15%) (Van der Ven et al., 1994; van der Ven et al., 1995). Conversion of N_4-acetyl-sulfamethoxazole and sulfamethoxazole-N_1-glucuronide back to parent sulfamethoxazole was experimentally observed in water and wastewater (Göbel et al., 2007; Plósz et al., 2010b; Radke et al., 2009; Stadler et al., 2015). Due to a low adsorptive polar nature, SMX is ubiquitously present in different environments with concentrations as high as 7910 ng L^{-1} (Peng et al., 2006) in raw influent and hundreds ng L^{-1} (Pin Gao et al., 2012; Rosal et al., 2010) in final effluent of WWTPs. The compound was categorized as Class 1: high priority pharmaceuticals relevant to the water cycle identified in a European assessment (de Voogt et al., 2009). Removal of SMX in WWTPs is marked by inconsistent results, but incomplete elimination was reported (Behera et al., 2011; Michael et al., 2013; Watkinson et al., 2009). For these reasons, there is an obvious need for efficient processes to enhance/complete elimination of SMX from WWTPs in order to prevent the risk of resistance bacteria and facilitate the reuse of wastewater. Bioaugmentation was proposed as an alternative technology to increase MPs removal efficiency in WWTPs by inoculating specialized degrading bacteria. Few studies of bioaugmentation for MP removal, in general, or SMX removal, specifically, have been reported. This study aimed at evaluating the potential for bioaugmentation as an advanced strategy for enhancing SMX removal from WWTPs by using microbial cultures that can degrade the compound.

Achromobacter denitrificans strain PR1 was selected for the bioaugmentation purpose of this study. The selection of this pure culture was based upon work of another partner, the Faculty of Engineering of the University of Porto (FEUP). The strain was isolated from activated sludge from a WWTP in the North of Portugal, characterized and demonstrated to have capability to degrade and use

SMX as source of carbon, nitrogen and energy, which is an uncommon property amongst *Achromobacter* spp (Reis et al., 2014).

The specific goals of this work are:

- To better understand the kinetics of sulfamethoxazole degraders in pure culture as well as upon bioaugmentation to activated sludge systems.
- To investigate the potential influence of retransformation processes of the two main human metabolites, i.e. N_4-acetyl-SMX and sulfamethoxazole-N_1-glucuronide, on the fate of sulfamethoxazole under activated sludge processes.
- To study the effect of redox potentials for wastewater treatment, e.g. aerobic and anoxic conditions, on the transformation rates of the three targeted compounds by both activated sludge and bioaugmented activated sludge with SMX degrading strains.
- To develop a bioaugmentation strategy for the continuous removal of SMX containing wastewater. A membrane bioreactor (MBR) was used to prevent washout of microbes, thus maintaining the survival and activity of the bioaugmented strain. The effect of operational conditions, e.g. HRTs, acetate as additional carbon source, SMX shock loading, on the removal of SMX was also tested.
- Develop and calibrate a model to describe the removal of the three compounds in the tested systems

An overview of all chapters is mentioned below:

Chapter 1 gives an introduction on MPs in the environment and the fate of these compounds in WWTPs as well as factors that could affect the removal of these compounds from WWTPs. Also, an overview of bioaugmentation as advanced technology for MPs removal from WWTPs is given.

Chapter 2 deals with characterization of kinetics of sulfamethoxazole degraders. A strain identified as *Achromobacter denitrificans* PR1 was previously isolated and found to be capable of using sulfamethoxazole (SMX) as a sole source of carbon, nitrogen and energy with the accumulation of 3-amino-5-methylisoxazole as degradation metabolite that is less toxic than the parent compound SMX. This chapter investigated the kinetics of sulfamethoxazole (SMX) degradation by *Achromobacter denitrificans* strain PR1 at a wide range of concentrations, from mg L^{-1}, μg L^{-1} to ng L^{-1} (environmentally relevant concentrations). The necessity for an additional carbon source, e.g. acetate and/or succinate for enhancing SMX degradation; and a comparison of the kinetics to literature values for WWTP sludge was performed to assess the feasibility of using the strain for bioaugmentation purposes.

In Chapter 3, the *A. denitrificans* strain PR1 was studied for bioaugmentation in suspended activated sludge process for enhancing SMX removal from wastewater. Different batch experiments were

conducted to also test (i) the potential of human metabolites, i.e. N_4-acetyl-SMX and sulfamethoxazole-N1-glucuronide, to convert back to parent SMX; (ii) necessity for supplementing with a biogenic substrate (e.g. acetate) to achieve a sufficiently interesting kinetic for SMX removal upon bioaugmentation with PR1; (iii) effect of redox conditions, i.e. aerobic and anoxic conditions, on the transformation rates of targeted compounds. A suitable mathematical model was applied in order to examine more the metabolic mechanism, as well as to predict the kinetics of SMX biotransformation and human metabolites retransformation in the bioaugmented and non-bioaugmented systems, under the different tested conditions.

In Chapter 4, bioaugmentation of *A. denitrificans* strain PR1 to MBR reactors were conducted to examine the potential for the ability and stability of the strain to degrade SMX over a long-term period. The influence of HRTs, supplementing of acetate as additional carbon source as well as shock loading on the reactor performances were also examined. A qPCR method targeting the strain-specific marker gene (Cas1), was developed for monitoring the survival of *A. denitrificans* in both bioaugmented and non-bioaugmented MBR reactors upon the bioaugmentation of PR1 for enhancing SMX removal from wastewater.

Finally, in Chapter 5, the obtained results are discussed within the framework of the research objective. Conclusions are drawn and perspectives for further research are proposed.

2

IMPACT OF BIOGENIC SUBSTRATE ON SMX BIODEGRADATION KINETICS BY *ACHROMOBACTER DENITRIFCANS* STRAIN PR1

Abstract

Pure cultures have been found to degrade pharmaceutical compounds. However, these cultures are rarely characterized kinetically at environmentally relevant concentrations. This study investigated the kinetics of sulfamethoxazole (SMX) degradation by *Achromobacter denitrificans* strain PR1 at a wide range of concentrations, from ng L^{-1} to mg L^{-1}, to assess the feasibility of using it for bioaugmentation purposes. Complete removal of SMX occurred for all concentrations tested, i.e. 150 mg L^{-1}, 500 µg L^{-1}, 20 µg L^{-1}, and 600 ng L^{-1}. The reaction rate coefficients (k_{bio}) for the strain at the ng L^{-1} SMX range were: 63.4±8.6, 570.1±15.1 and 414.9±124.2 L.gXss^{-1}.d^{-1}, for tests fed without a supplemental carbon source, with acetate, and with succinate, respectively. These results were significantly higher than the value reported for non-augmented activated sludge (0.41 L.gX_{ss}^{-1}.d^{-1}) with hundreds of ng L^{-1} of SMX. The simultaneous consumption of an additional carbon source and SMX suggested that the energetic efficiency of the cells, boosted by the presence of biogenic substrates, was important in increasing the SMX degradation rate. The accumulation of 3-amino-5-methylisoxazole was observed as the only metabolite, which was found to be non-toxic. SMX inhibited the *V. fischeri* luminescence after 5 min of contact, with EC_{50} values of about 53 mg L^{-1}. However, this study suggested that the strain PR1 still can degrade SMX up to 150 mg L^{-1}. The results of this work demonstrated that SMX degradation kinetics by *A. denitrificans* PR1 compares favorably with activated sludge and the strain is a potentially interesting organism for bioaugmentation for SMX removal from polluted waters.

Keywords: wastewater; antibiotics; sulfonamide; co-metabolism; bioaugmentation; ecotoxicity

Published as: Nguyen, P. Y., Carvalho, G., Reis, A. C., Nunes, O. C., Reis, M. A. M., & Oehmen, A. (2017). "Impact of biogenic substrates on sulfamethoxazole biodegradation kinetics by *Achromobacter denitrificans* strain PR1". *Biodegradation*, 1-13. doi: 10.1007/s10532-017-9789-6

2.1. Introduction

The term antibiotic is used to denote a chemotherapeutic agent that inhibits or abolishes microbes by specific interactions with bacterial targets. In 2013, the population-weighted EU/EEA mean consumption of antibiotics was 22.4 defined daily doses (DDD) per 1000 inhabitants per day, representing a continuing increase over the last five years for the EU as a whole (ECDC, 2014). Antibiotics have been reported to contaminate the natural environment in many countries in Europe, North America and Asia and sulfamethoxazole (SMX) is ubiquitously present, having been detected in river water (Batt et al., 2006a; Gonzalez-Pleiter et al., 2013; Kolpin et al., 2002; Tamtam et al., 2009; Watkinson et al., 2009; Xu et al., 2007), groundwater (Hirsch et al., 1999; Lindsey et al., 2001; Sacher et al., 2001), drinking water (Zuccato et al., 2000), sediments (Kerry et al., 1996; Kim et al., 2009), biota (Kong et al., 2007) and WWTP effluents (Batt et al., 2006a; Costanzo et al., 2005; Panpan Gao et al., 2012; Gros et al., 2010; Rosal et al., 2010; Watkinson et al., 2009). Concern regarding the environmental presence of sulfonamides and other species of antibiotics has focused mainly on the potential spread of antimicrobial resistance due to extended exposure, even at relatively low concentrations (Dantas et al., 2008). Urban wastewater treatment plants (WWTPs) are considered as one of the main 'hotspots' of potential evolution and spreading of antibiotic resistance into the environment (Manaia et al., 2016).

Among antibiotics, sulfonamides constitute one of the most consumed antimicrobial families and SMX (whose structure and physical-chemical properties are provided in Table A3 – Appendix A3) is one of the most widely used synthetic sulfonamide antibiotics worldwide (Kumar and Xagoraraki 2010). SMX prevents the formation of dihydrofolic acid, a compound that bacteria must be able to produce in order to survive. SMX was also found to pose an ecological risk to aquatic ecosystems (Eguchi et al., 2004; Gros et al., 2010; Isidori et al., 2005; Nunes et al., 2005; Park and Choi, 2008). SMX is a low adsorptive, polar, sulfonamide antibiotic, thus its fate in aqueous environments is of high concern. SMX decrease in WWTPs' effluents is mainly due to microbial activity (Müller et al., 2013). SMX removal was observed to be dependent on the wastewater treatment processes, and typically incomplete. Most of the studies on biodegradation of SMX looked at the removal of these compounds by activated sludge (Collado et al., 2013; Drillia et al., 2005a; Müller et al., 2013; Yu et al., 2009). It is unanimous that efficient measures should be urgently considered to eliminate sulfonamides from WWTPs in order to facilitate the safe discharge and potential reuse of wastewater.

Bioaugmentation can be an alternative to increase MP removal efficiency in WWTPs, by inoculating specialized xenobiotic degrading bacteria (Van Limbergen et al., 1998). Up to now, the microbial transformation of SMX has been reported in some bacterial strains, mainly belonging to the genus *Microbacterium* (Ricken et al., 2013), *Pseudomonas* (Jiang et al., 2014) and *Achromobacter* (Bouju et al., 2012). But so far, degradation of sulfonamides at environmentally relevant concentrations has not been

tested with these bacterial strains. This study focused on *A. denitrificans* PR1, previously isolated from activated sludge from a WWTP in the North of Portugal, which has the capability to use sulfamethoxazole as the sole source of carbon, nitrogen and energy, with stoichiometric accumulation of a metabolite, 3-amino-5-methylisoxazole (Reis et al., 2014). Previous studies with this strain, as is this the case for many pure culture studies, were carried out using relatively high concentrations of SMX (at the mg L^{-1} level). The aim of this work was to determine the kinetics of SMX degradation by strain PR1 at lower SMX concentration ranges to assess the feasibility of using it for bioaugmentation in WWTPs. It is hypothesized that at the low concentration levels of SMX normally found in wastewater (µg L^{-1} to ng L^{-1}), the contaminant concentration is too low to induce the catabolic genes (Kolvenbach et al., 2014), and thus biodegradation may be dependent on co-metabolism, in the presence of another carbon source available at higher concentration. An alternative mechanism previously reported for other xenobiotic compound (Chong and Chiou, 2010; Egli, 2010; Oehmen et al., 2013) is a dependency on the energy generated through the metabolism of biogenic substrates. For the purposes of this study, succinate and acetate were chosen as additional, easily biodegradable substrates, since the same strain was previously shown to use succinate as a growth substrate (Reis et al., 2014) and acetate is a substrate that is frequently present in wastewater treatment plants.

The removal of the selected antibiotic by *A. denitrificans* strain PR1 and its biodegradation metabolites under different conditions were investigated in order to characterize the SMX biodegradation capacity of the culture through assessing (i) the SMX degradation dependency on an additional carbon source, to understand the biodegradation mechanism involved; and ii) the kinetics of SMX degradation at low concentration ranges and in presence/absence of acetate/succinate, comparing it to literature values for WWTP activated sludge, to study the strain's feasibility as a culture that can be applied for bioaugmentation.

2.2. Materials and Methods

2.3.1. Chemicals and reagents

Reagent grade (purity ≥ 99%) sulfamethoxazole, sulfanilic acid and 3-amino-5-methylisoxazole were purchased from Sigma-Aldrich. The isotopically labelled D4-sulfamethoxazole was obtained from Toronto Research Chemicals (TRC, Canada). All other reagents were of analytical grade from commercial sources. Individual stock standard solutions were prepared on a weight basis in methanol and stored at -20ºC. A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. HPLC-grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany).

2.3.2. Analytical procedure

For the levels of mg L^{-1} and 500 µg L^{-1} of sulfamethoxazole tested, the concentration of SMX, sulfanilic acid and 3-amino-5-methylisoxazole was determined by direct injection using a Waters system equipped with ultraviolet (UV) and fluorescence detectors (Waters Chromatography, Milford, MA, USA).

For the lower levels of SMX tested, the concentration of SMX and 3-amino-5-methylisoxazole was determined by direct injection using a high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific.

A detailed description of the analytical methods used is provided as Apppendix A.

The acetate and succinate concentrations were determined by high-performance liquid chromatography (HPLC) using an IR detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.6 mL/min and a 50^{o}C operating temperature.

2.3.3. SMX degradation by A. denitrificans strain PR1

SMX degradation at concentrations of mg L^{-1}, µg L^{-1} and ng L^{-1} were conducted to determine the effect of SMX concentration levels on the mechanism of SMX degradation by strain PR1. Experiments were performed to: (i) confirm the ability of strain PR1 to degrade SMX in the absence of additional sources of carbon and energy; (ii) evaluate the effect of acetate or succinate as a source of supplemental carbon and energy on the degradation rate; (iii) evaluate the fractions of total degradation which could be attributed to adsorption and biodegradation. Experiment 1 assessed SMX degradation with no additional carbon source added, experiment 2 contained additionally 0.59 g L^{-1} acetate, experiment 3 contained 0.59 g L^{-1} succinate instead of acetate, as additional C-source, and in experiment 4, NaN_3 (2 g L^{-1}) was added to stop bacterial activity and assess SMX adsorption. For the concentration of 150 mg L^{-1} of SMX tested, experiment 3 was supplemented with yeast extract (YE) (0.2g L^{-1}) as source of vitamins. All the tests were conducted in mineral medium B (Barreiros et al., 2003), with 0.5 g L^{-1} ammonium sulfate as nitrogen source (here designated MMBN). For all of the SMX biodegradation experiments, the cells were harvested by centrifugation (15 min, 7000 rpm in a Sigma® 4-16KS centrifuge), washed twice with saline solution (NaCl, 8.5 g L^{-1}) and once with MMBN. Then the pellet was resuspended in MMBN to get an initial cell suspension concentration ranging from 0.02-0.09 g biomass L^{-1}. Growth was monitored with optical density measurement at 600 nm using Hatch Lange DR 2800 spectrophotometer. Biomass concentration was estimated from a correlation of optical density (OD_{600nm}) to dry weight. For strain *A. denitrificans* PR1, biomass concentration (mg L^{-1}) = OD_{600nm} * 0.526 g biomass L^{-1} (Reis et al., 2014).

Tests at SMX levels of 150 mg L^{-1} and 500 µg L^{-1}, 20 µg L^{-} and 600 ng L^{-1}

All tests were conducted in 500 mL Erlenmeyer flasks. The final volume (after addition of the inoculum) for each test was 200 mL,where SMX was fed at 150 mg L^{-1}, 500 µg L^{-1}, 20 µg L^{-1} and 600 ng L^{-1}, with and without addition of acetate (10 mM) and/or succinate (5 mM) as additional C-sources. All experiments were performed in duplicate.

Cultures of 150 mg L^{-1} and 500 µg L^{-1} were incubated at 30°C on a shaker at 150 rpm. At regular intervals, 6 mL samples were taken for SMX quantification, which was carried out using a Waters system equipped with ultraviolet (UV) and fluorescence detectors (Waters Chromatography, Milford, MA, USA).

The assays of 20 µg L^{-1} and 600 ng L^{-1} were carried out at 20°C. 6 mL samples were frequently taken for SMX quantification using a high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific.

Experiments were also performed to assess paired substrate competitive inhibition on SMX degradation at 20 µg L^{-1} of SMX. In this case, succinate and acetate were spiked at a concentration of 2.5 and 5 mM, respectively.

2.3.4. Determination of kinetic parameters

The kinetics of SMX is described with Equation 2.1 (Joss et al., 2006b):

$$\frac{dS}{dt} = -k_{bio} X_{SS} S \tag{2.1}$$

Where, S is the soluble compound concentration (g L^{-1}), t is the time (d), k_{bio} the reaction rate constant ($Lg_{SS}^{-1}d^{-1}$), and X_{SS} the suspended solids concentration (g L^{-1}). The software Aquasim was used to simulate the SMX removal and estimate the reaction rate coefficient, k_{bio}.

The specific growth rate was calculated using Equation 2.2.

$$X = X_0 e^{\mu t} \tag{2.2}$$

where t represents time, X biomass concentration and X_0 biomass concentration at t=0. The biomass yield (Y) was determined through Eq. (2.3):

$$Y = \frac{\Delta X}{\Delta S} = \frac{X_t - X_0}{S_0 - S_t} \tag{2.3}$$

where t represent the time, S_t and S_0 represent the substrate content at time t and t = 0, respectively. X_t is biomass concentration at time t and X_0 is biomass concentration at t = 0.

For the batch test performed at 30ºC, the specific growth rate at 20ºC was calculated based on the Q_{10} model (van't Hoff, 1884):

$$Q_{10} = \left(\frac{\mu_2}{\mu_1} \right)^{10/(\theta_2 - \theta_1)} \tag{2.4}$$

where μ_2 and μ_1 are specific growth rates at 2 temperatures, θ_2 and θ_1, respectively. Many processes follow this model, often with a Q_{10} near 2 (Cossins Bowler, K., 1987).

2.3.5. Ecotoxicity tests

The freeze-dried luminescent bacteria (*Vibrio fischeri* DSM 507) were purchased from DMSZ (Braunschweig, Germany). *V. fischeri* was reactivated in Zobell Marine Broth (Himedia) and the biomass was cryopreserved on glycerol (15%, v/v) at -80°C. Toxicity of both SMX and its main metabolite (3A5MI) towards *V. fischeri* was assessed according to the Standard Microtox® Procedure (EN ISO/DIS 11348-3). A single colony of *V. fischeri* previously grown on Marine Broth Agar was used to inoculate Marine Broth and the culture was incubated at 20°C for 20 h, 120 rpm. The biomass was collected by centrifugation and resuspended in Microtox® reconstitution solution.

SMX and 3A5MI standards were prepared in distilled water and sterilized by filtration (nylon syringe filters, 0.22 µm, 25 mm, VWR). The range of SMX and 3A5MI concentrations used were 0.7 – 76.0 mg L^{-1} (2.8-300 µM) and 0.3 – 88.3 mg L^{-1} (2.8-900 µM), respectively. Tests were performed on 96-well microplates and incubated at 15°C and 120 rpm. Luminescence measurements were performed using Synergy HT Multi-Mode Microplate Reader (Biotek Instruments, USA).

EC_{50} and EC_{20} values, respectively representing the effective concentration necessary to reduce half or 20% of the initial luminescence, were calculated according to the standard procedure after 5 min of contact.

2.3. Results

2.3.1. Degradation of SMX and biogenic substrates by A. denitrificans *strain PR1*

In this study, batch tests were initially carried out at a concentration of 150 mg L^{-1} of SMX to determine the kinetics using the conditions employed for pre-culturing (Figure 2.1). For the level of 150 mg L^{-1} SMX, the removal of the antibiotic with inactivated biomass (experiment 4) was 8% (data not shown). In experiment 1, in the absence of other carbon source, almost no biomass growth was ob-

served (Figure 2.1), but approximately 91% of SMX was degraded by strain PR1 after 51 hours, ruling out co-metabolism. In the presence of additional carbon source, two patterns of SMX degradation could be observed: (i) simultaneous degradation of succinate and SMX could be observed in the first 23.5 hours, (ii) followed by faster SMX degradation during the remaining period after succinate had been completely consumed (Figure 2.1). Furthermore, as shown in Figure 2.2, 3-amino-5-methylisoxazole (3A5M) was observed to be the main metabolite identified, as the other biodegradation metabolite (sulfanilic acid) was not observed to accumulate.

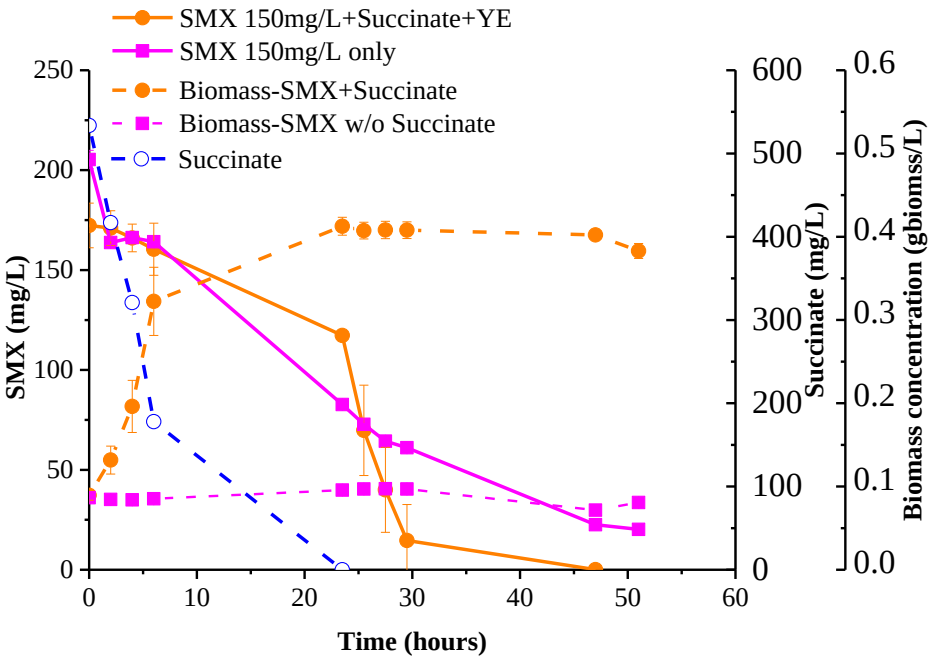


Figure 2.1. Relative change of SMX concentration, biomass concentration and C-sources over time by *A. denitrificans* strain PR1 in mineral medium B supplemented with 150 mg L^{-1} of SMX in the presence or absence of other carbon source (0.59g L^{-1} of succinate)

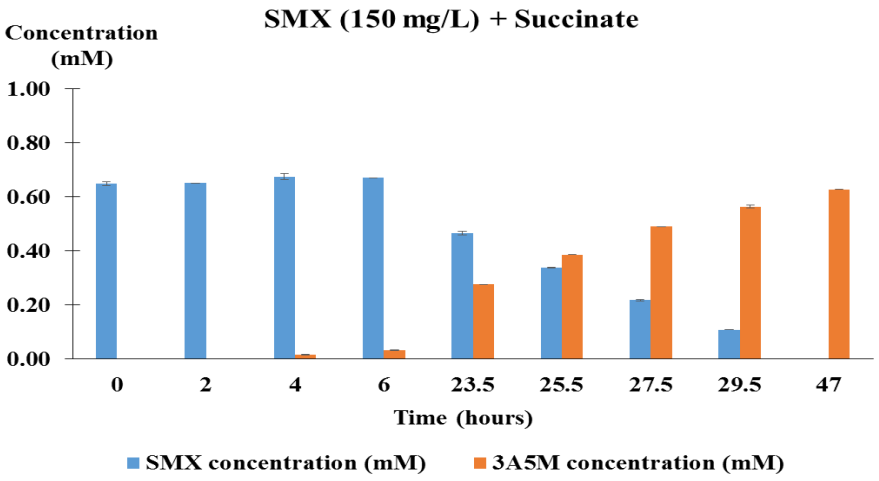


Figure 2.2. Accumulation of 3-amino-5-methylisoxazole during degradation of SMX by A. denitrificans strain PR1. Bars represent standard deviation of two independent experiments

The tests carried out with concentrations closer to the values found in wastewater showed a different behavior, where the removal of the biogenic substrate did not precede SMX degradation, but both compounds were removed either simultaneously (Figure 2.3), or the biogenic substrate was even removed after SMX (Figures 2.4 and 2.5). Furthermore, the SMX removal increased significantly in the presence of acetate/succinate (Figures 2.3, 2.4 and 2.5). This effect was observed with both biogenic substrates when fed individually, and also when both compounds were added simultaneously.

For the tests fed with 500 µg L^{-1}, SMX showed a removal of 44.5±8.8%, 90±2% and 2.4±0.6% of SMX in the experiments which consisted of only SMX, of supplementing with 10 mM of acetate as additional carbon source, and in the control, respectively. These results suggest that the higher SMX degradation by strain PR1 is either due to higher energetic efficiency with the biogenic substrate or due to the increase in biomass concentration. However, in the batch tests fed with 600 ng L^{-1} and 20 µg L^{-1} of SMX, there was a removal of >90% of SMX in the first 4 hours and 8.5 hours in the tests with acetate and succinate, respectively, while negligible biomass growth was observed during this period. In contrast, in the absence of additional carbon source, only about 71% and 46 % of SMX was removed after 8.5 hours for the concentration of 600 ng L^{-1} and 20 µg L^{-1} of SMX, respectively (Figures 2.4A and 2.5A). These results suggest that the presence of the biogenic substrate led to the increased SMX removal by strain PR1.

A **B**

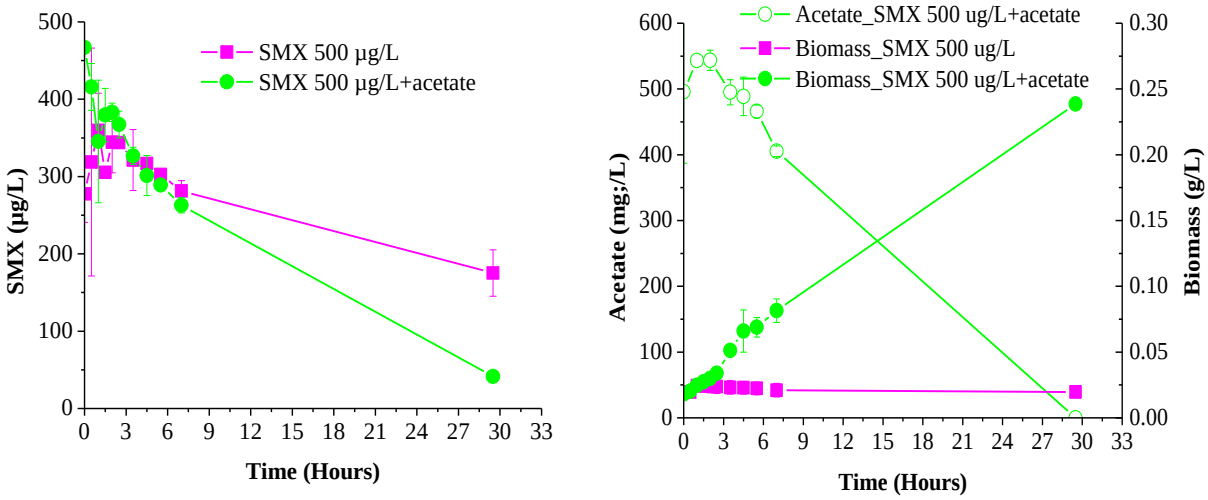
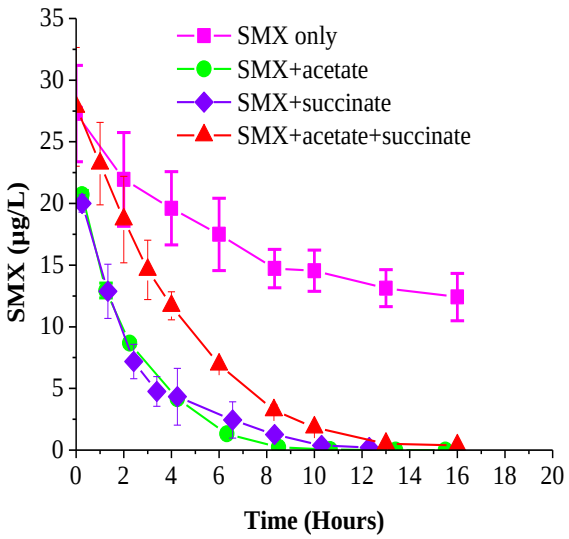
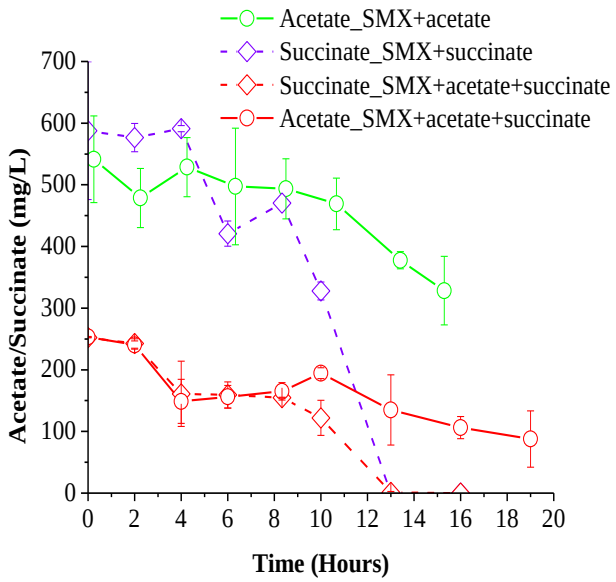


Figure 2.3. Relative change of SMX concentration (a) and biomass content and C-sources (b) over time by *A. denitrificans* strain PR1 in MMBN supplemented with 500 µg L^{-1} of SMX in the absence of a supplemental carbon source (acetate)

A



B



C

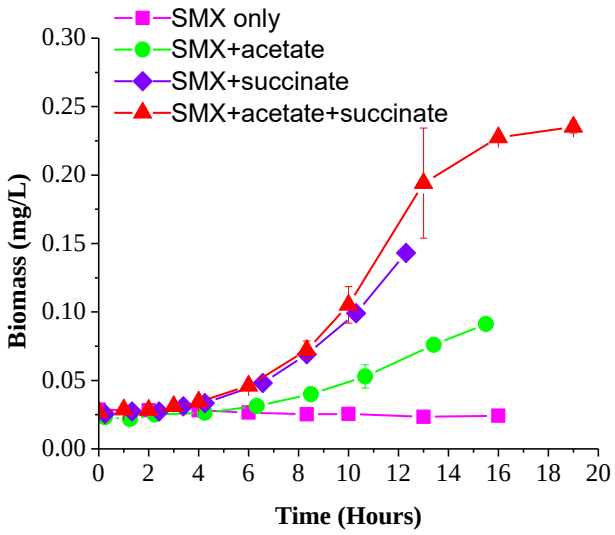


Figure 2.4. Relative change of SMX concentration (a), C-sources (b) and biomass content (c) over time by *A. denitrificans* strain PR1 in MMBN supplemented with 20 µg L^{-1} of SMX in the absence or presence of a supplemental carbon source, i.e. acetate or succinate

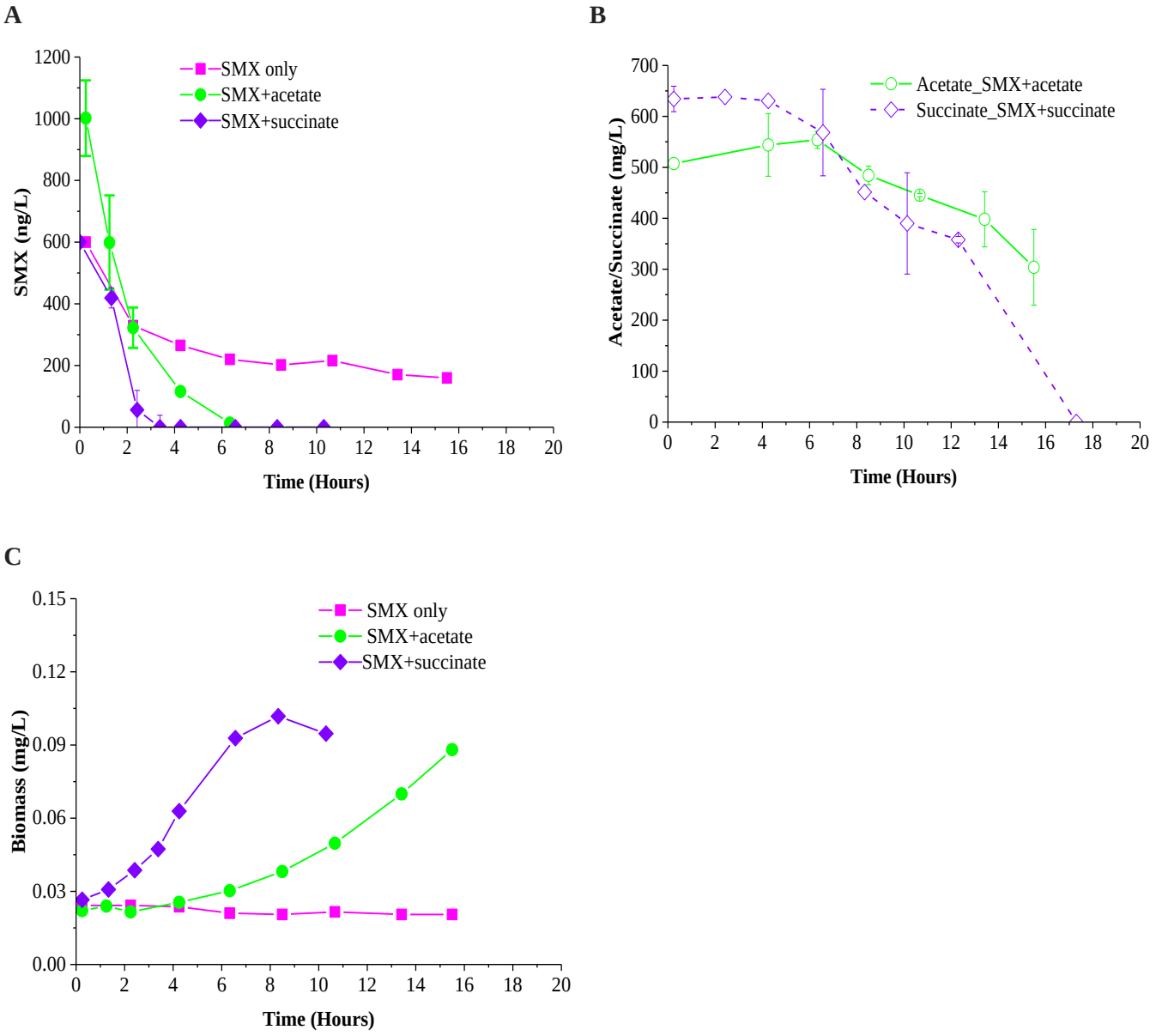


Figure 2.5. Relative change of SMX concentration (a) and C-sources (b) and biomass content (c) (over time by *A. denitrificans* strain PR1 in MMBN supplemented with 600 ng L^{-1} of SMX in the absence or presence of a supplemental carbon source, i.e. acetate or succinate

2.3.2. Modelling cellular growth and SMX degradation of A. denitrificans *strain PR1*

The average specific growth rate of strain PR1 was determined for the concentrations of 600 ng L^{-1}, 20 µg L^{-1} (Table 2.1) using either succinate or acetate as growth substrate. It was found that growth was not inhibited by SMX at these low levels. SMX was removed prior to biogenic substrate consumption and the growth on acetate and succinate did not seem affected by the initial exposure to SMX in the ng L^{-1} to µg L^{-1} range. The specific growth rate was higher for succinate vs acetate as well as the yield of biomass grown on succinate vs acetate Table 2.1.

The kinetic rate coefficients of SMX degradation were determined using the data obtained at different SMX concentrations. Figure 2.6 shows the experimental results and the model simulations of SMX degradation for the tests fed with 20 µg L^{-1}, and 600 ng L^{-1} of SMX in the presence of acetate or succinate. Good agreement between the experimental data and model simulations indicate that the first order kinetics expressed by Equation 1 represents the data well, except for the case of SMX in the absence of an additional C-source (Figure 2.6). In this case, the biodegradation tendency deviates slightly from first order kinetics, since the reaction rate slows down after removal of approximately 50% of the SMX. Perhaps the lack of biogenic substrate caused there to be a limited amount of energy available in the cells to degrade all of the SMX present.

Table 2.1. Specific growth rate (µ) and biomass yield (Y) of *A. denitrificans* strain PR1 in MMBN in the presence of 5mM of succinate or 10 mM of acetate

	SMX (600 ng L^{-1}) with		**SMX (20 µg L^{-1})**	
	Succinate	Acetate	Succinate	Acetate
µ (d^{-1})	3.73±0.04	2.22±0.02	3.71±0.04	2.30±0.12
Y ($g_{cell\ dry\ weight}/g_{substrate}$)	0.396±0.013	0.236±0.003	0.445±0.050	0.325±0.022

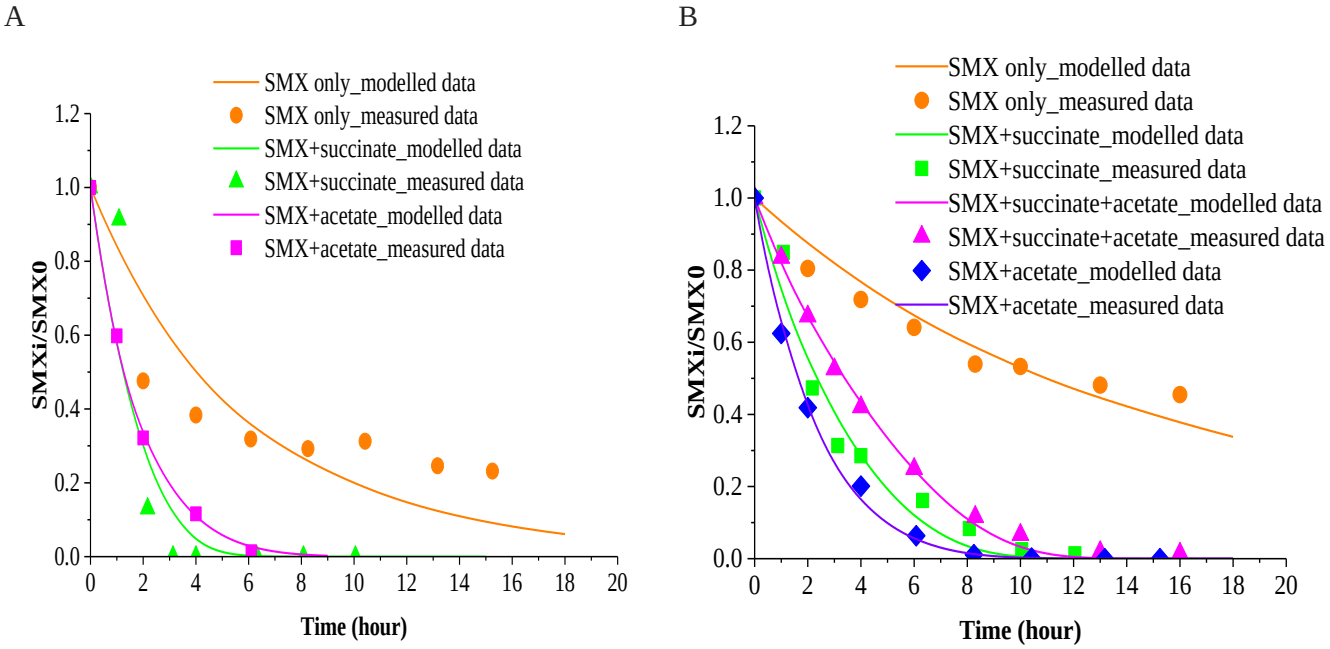


Figure 2.6. Measured and modeled data of SMX degradation at 20 µg L^{-1} of SMX (A) and 600 ng L^{-1} of SMX (B) in the aerobic batch experiment in the presence of acetate/succinate. SMXi and SMX0 indicates the SMX concentration at time i and time 0, respectively

A summary of the kinetic reaction rate coefficients is shown in Table 2.2 for SMX degradation by *A. denitrificans* strain PR1 in the presence and absence of additional carbon sources at different concentration levels. It is clear from Table 2.2 that the first order reaction rate coefficients (k_{bio}) increased significantly in the presence of an additional easily biodegradable substrate or mixed substrates. It was observed though that the reaction rate coefficient in the test fed with both succinate and acetate substrates was lower than for the tests fed with acetate or succinate individually. This suggests that there occurred a competition for substrates when multiple carbon sources are fed simultaneously.

The results demonstrated that the kinetics of SMX degradation by *A. denitrificans* strain PR1 in this study compares favorably with activated sludge from other studies (Table 2.2). The k_{bio} of the culture was about 2 and 3 orders of magnitude higher for tests fed without and with a supplemental carbon source (acetate/succinate), respectively, than that of non-augmented activated sludge of 0.41 L gX_{ss}^{-1} $.d^{-1}$ (Plósz et al., 2010b) and 0.3 L gX_{ss}^{-1} d^{-1} (Suárez et al., 2010).

Table 2.2. Reaction rate coefficient (k_{bio}) for SMX degradation by *A. denitrificans* strain PR1 and comparison with other biological systems

Biomass	Matrix	SMX concentration	k_{bio} (L gX_{ss}^{-1} d^{-1})	Reference
A. denitrificans	Media w/o other C-source	600 ng L^{-1}	63.4±8.6	This study
A. denitrificans	Media with acetate	600 ng L^{-1}	570.1±15.1	This study
A. denitrificans	Media with succinate	600 ng L^{-1}	414.9±124.2	This study
A. denitrificans	Media w/o other C-source	20 µg L^{-1}	56.2±3.7	This study
A. denitrificans	Media with acetate	20 µg L^{-1}	445.6±24.2	This study
A. denitrificans	Media with succinate	20 µg L^{-1}	372.0±24.3	This study
A. denitrificans	Media with mixed substrates of acetate and succinate	20 µg L^{-1}	270.4±29.2	This study
Activated sludge (AS)	Preclarified wastewater	800 ng L^{-1}	0.41	(Plósz et al., 2010b)
Nitrifying aerobic AS	Synthetic feed	20 µg L^{-1}	0.3	(Suárez et al., 2010)

2.3.3. Toxicity tests for SMX and biodegradation metabolite 3A5MI

The bioaugmentation process must ensure that the target pollutant is degraded into harmless compounds. Given *A. denitrificans* strain PR1 degrades SMX with the stoichiometric accumulation of 3A5MI, the toxicity of this metabolite was assessed, and compared to that of the parent compound. No

inhibition of the *V. fischeri* luminescence occurred, even when the 3A5MI concentration was 1.5 times higher than that formed in the 150 mg L^{-1} SMX assays. In contrast, SMX inhibited the *V. fischeri* luminescence after 5 min of contact, with EC_{50} values of about 53 mg L^{-1} (~200 µM) (Table 2.3).

Table 2.3. Average EC_{50} and EC_{20} values of SMX and 3A5MI

Reference compound	Concentration range (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})
SMX	0.7-76.0	52.7	23.9
3A5MI	0.3-88.3	No inhibition	No inhibition

2.4. Discussion

The removal of SMX in biological processes could potentially be due to abiotic losses (i.e. volatilization, hydrolysis), biodegradation or bio-sorption. However, low removal of SMX was observed in the control tests during the experimental period. Indeed, a low Henry's Law constant ($6.4x10^{-13}$ atm.m^3/mol (Kimura et al., 2004)) indicates that SMX is expected to be essentially nonvolatile from water surfaces, which was corroborated in previous studies (Li and Zhang, 2010; Perez et al., 2005). Moreover, SMX was found to have a low solid-water distribution coefficient (K_d) (Carballa et al., 2008), ranging between a log K_d of 0.8 and 1.8 in digested sludge at different operational conditions, indicating that this compound does not sorb onto sludge to an appreciable extent. The n-octanol water distribution coefficient (K_{ow}) of SMX also had a low value (log K_{ow} of 0.89 (Kolpin et al., 2002)), (ECDC (2014) indicating that the lipophilic interactions of SMX with the lipid fraction of sludge (i.e. absorption) should not be significant. The compound could also be adsorbed onto the surface of microorganisms due to the establishment of electrostatic interactions. SMX exhibits a positively charged amino group at a pH <pKa_1 (1.9), and a negatively charged conjugate due to the loss of the sulfonamide proton at pH > pKa_2 (5.7). Thus, the test conditions did not favor the interactions between SMX and the negatively charged surface of microorganisms. Indeed, the results of the tests performed with inactivated biomass in this study confirmed that abiotic processes had negligible contribution to SMX removal.

A. denitrificans strain PR1 was found to be capable of mineralizing the aniline moiety of SMX with the stoichiometric accumulation of 3-amino-5-methylisoxazole (3A5MI) (Reis et al., 2014), which lacks antimicrobial activity, and no other metabolites were detected in this study. This is consistent with previous results (Reis et al., 2014). To date, only few data on SMX metabolites by biological processes were reported. Possible SMX biodegradation pathways and postulated metabolites were identified using the EAWAG-BBD Pathway Prediction System (EAWAG-BBD) (Figure 2.7). Müller et al., (2013) reported 3-amino-5-methylisoxazole as the main metabolite when SMX was supplied as a co-substrate in an activated sludge process (bench scale) and hydroxyl-N(5-methyl,2-oxazle-3-yl)benzene-1-sulfonamide as a further metabolite when SMX was provided as sole carbon and nitro-

gen source. In a study by Gauthier et al., (2010), hydroxyl-N(5-methyl,2-oxazole-3-yl)benzene-1-sulfonamide was detected as stable metabolite when SMX was degraded by a consortium of fungi and *Rhodococcus rhodochrous*. In another study, aniline, 3A5MI, 4-aminothiophenol and sulfanilamide were the major intermediates from sulfamethoxazole biodegradation by strain *Pseudomonas psychrophila* HA-4 (Jiang et al., 2014). In the current study, it was demonstrated that the metabolite accumulated by strain PR1 does not inhibit the luminescence of *V. fischeri*. 3A5MI was shown to be less toxic to *V. fischeri* than SMX, which had an EC_{50} of approximately 53 mg L^{-1} at a contact time of 5 min. Moreover, the EC_{50} calculated for 3A5MI for *Daphnia magna* was 100 mg L^{-1} (Trovó et al., 2009), showing that 3A5MI is also not toxic at a higher trophic level at the concentrations formed in this study. Furthermore, this intermediate product has been found to be effectively biodegraded in other processes, such as microbial fuel cells (Wang et al., 2016). The fact that 3A5MI possesses lower toxicity than SMX is more important when assessing an organism such as *A. denitrificans* PR1 for potential bioaugmentation applications, since situations of increased toxicity caused by treatment processes should clearly be avoided.

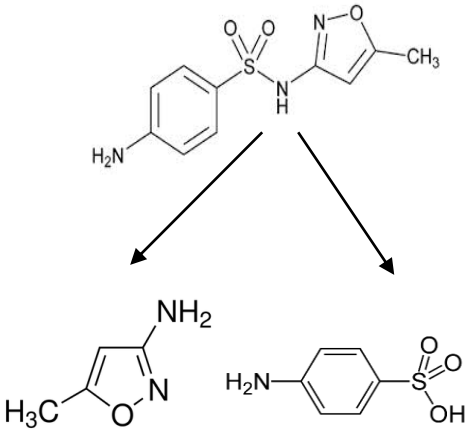


Figure 2.7. Predicted transformation products of sulfamethoxazole in EAWAG-BBD

In the tests conducted with only SMX addition, *A. denitrificans* PR1 had the ability to degrade SMX even in the absence of supplemental carbon substrate, succinate/acetate. In a previous study with *A. denitrificans* PR1, SMX was also shown to be degraded in the absence of other carbon source at the mg L^{-1} range (Reis et al., 2014), which is consistent with the results of this study (Figure 2.1). These results show that the mechanism of SMX biodegradation by *A. denitrificans* PR1 does not depend on co-metabolism in the presence of a primary substrate. In the presence of other growth substrate, this high concentration of SMX was degraded completely below limit of detection (LOD) levels, but there

was a lag phase of SMX consumption at the beginning of the test and the SMX degradation was enhanced after the succinate was completely consumed. The initially slower rate of SMX degradation in the presence of another carbon source could be attributed to a competition effect (Reis et al. 2014) and the fact that strain PR1 prefers taking up succinate, a readily biodegradable carbon source, as compared to the antibiotic SMX.

At the low levels of SMX, the removal rate of the antibiotic was increased in the presence of a biogenic substrate (acetate/succinate) or a mixture of both biogenic substrates. The consumption of acetate or succinate followed that of SMX in these tests suggesting that, at these concentration ranges, the presence of other carbon sources did not hinder the SMX degradation rate but that PR1 preferred to degrade SMX than the biogenic substrate.

In most cases, at these low SMX levels, the degradation of SMX occurred at the beginning of the experiment without lag-phase, where no biomass growth was observed. Thus, the removal of SMX observed could not be justified by the increase in biomass concentration. Furthermore, the fact that SMX degradation occurred even in the absence of supplemental carbon source (acetate/succinate), again showed that SMX biodegradation was not due to co-metabolism. It is thus hypothesised that the supplemental carbon source (acetate/succinate), being of a biogenic nature, led to an increase in ATP production within the cell, which was important in increasing the SMX degradation rate. Chong et al., (2012) reported that biogenic substrates benefits activated sludge's acclimation and degradation of a xenobiotic by enriching the energy contents of the sludge cells by shortening acclimation lag phase and enhancing the xenobiotic degradation rate. The author observed positive relation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) conversion rate with ATP and negative relation of lag time with ATP which indicate that the biomass acclimation and degradation rate is most predominantly dependent on the energy contents of the sludge cells. This discovery also consolidates our hypothesis. The presence of biogenic substrates could be an important factor at low levels of SMX concentration (ng to µg L^{-1}), even in a pure culture capable of SMX degradation without other carbon sources, either to increase the rate of SMX transport across the cell membrane, or acting instead as a supplemental energy source and primary electron-donor substrate that augments the metabolic efficiency of SMX removal. Biogenic substrates could exert a complex set of interrelated effects during the degradation of xenobiotic compounds, i.e. acting as a growth inducer, reducing power regenerant, or an additional source of ATP. It is known that certain enzymes of microorganisms are formed only in the presence of specific substrates (Jacob J., 1961), supplying energy and building blocks for protein synthesis (Egli, 2010). The presence of biogenic substrates has been previously found to augment the degradation kinetics of other xenobiotic compounds, such as herbicides (Chong and Chiou, 2010; Oehmen et al., 2013), which is consistent with the results of this study.

To the author's best knowledge, the kinetics of SMX removal with and without the presence of biogenic substrates at environmentally relevant concentrations has not been reported yet for promising isolates that have been found to be capable of SMX degradation. Usually, the total biodegradation of SMX by strains that were isolated from activated sludge were reported over a period of one or more days at the mg L^{-1} range (Bouju et al., 2012; Herzog et al., 2013; Jiang et al., 2014). The results of this work clearly showed that the SMX concentration range had a large impact on the kinetics of SMX degradation by *A. denitrificans* PR1, where competition for carbon source ceased to be relevant when moving from the mg L^{-1} to ng L^{-1} or µg L^{-1} ranges. This highlights the importance of testing the kinetics and behavior of xenobiotic removal by pure cultures at environmentally relevant concentration ranges, in this case, similar to those observed in WWTPs.

SMX removal efficiencies in conventional WWTPs can vary from high 60% to 95% or completely removed (Carballa et al., 2004; Drillia et al., 2005a; Göbel et al., 2007; Li and Zhang, 2011; Miège et al., 2009; Peng et al., 2006; Xu et al., 2007; Yang et al., 2005; Yu et al., 2009), to low removal efficiencies of 20-24 % (Brown et al., 2006; Rosal et al., 2010; Ternes et al., 2007). The variation of SMX removal could probably be attributed to the de-conjugation of metabolites or the differences in wastewater treatment plant operation conditions, such as HRT, or the presence of an anaerobic compartment, and also to differences in the bacterial community composition, in particular the density of SMX degraders among different WWTPs (Halling-Sørensen et al. 1998; Michael et al. 2013). The wide range of SMX removal efficiencies of activated sludge underlies the variability in the abundance of organisms in WWTPs that are capable of degrading this important antibiotic. SMX biodegradation under aerobic conditions using conventional activated sludge showed that (at least some of the members of) the microbial community, composed of autotrophic nitrifying bacteria and heterotrophic bacteria, utilized SMX as energy, carbon and/or nitrogen source for growth (Müller et al., 2013). Nevertheless, the fact that this compound is widely present in wastewater, and rarely fully removed from WWTPs, shows that alternative WWTP operational strategies, such as bioaugmentation, are needed to reduce the unwanted releases of antibiotics into the environment.

The results of this study highlight the potential in applying *A. denitrificans* PR1 for bioaugmentation in wastewater treatment plants (WWTPs). Indeed, the k_{bio} reaction rate coefficients of SMX were found to be 2-3 orders of magnitude higher than that typically observed in activated sludge WWTPs. Since the goal of bioaugmentation is to accelerate the removal of undesired compounds from wastewater, the possibility of applying *A. denitrificans* PR1 could only be considered if SMX is degraded much more efficiently than in activated sludge communities, which was indeed the case in the present study. Acetate, one of the biogenic substrates applied in this work that augmented the SMX degradation rates, is typically present in WWTPs, which could help to stimulate the activity of *A. denitrificans* PR1 during application. Moreover, the specific growth rate (µ) of *A. denitrificans* PR1 with

acetate (2.2-2.3 d^{-1}) was observed to be higher than that of some other organisms relevant to WWTPs (Gujer et al., 1995), including autotrophic nitrifiers and polyphosphate accumulating organisms (PAOs). This suggests that *A. denitrificans* PR1 has the potential to establish itself in activated sludge communities and survive in real WWTPs, as these systems are often operated to achieve biological nutrient removal of nitrogen and phosphorus that promote the survival of nitrifiers and PAOs. Future work should address the feasibility of bioaugmenting activated sludge with *A. denitrificans* PR1, in order to achieve increased SMX removal in wastewater treatment systems.

2.5. Conclusions

A. denitrificans PR1 is able to degrade SMX in the presence or absence of biogenic substrates over a wide range of concentrations. The organism was observed to mineralize the aniline moiety of SMX with the accumulation of 3A5MI, which did not possess antimicrobial activity and had low toxicity. The results from pure culture tests show that the kinetics of SMX degradation by *A. denitrificans* PR1 compares favorably with activated sludge at the levels of ng L^{-1} that are typical of wastewater, presenting even higher kinetics in the presence of one or more supplemental carbon sources. It is thus concluded that biogenic substrates augment the energetic efficiency of the *A. denitrificans* PR1 cells at low levels of SMX concentration (µg L^{-1} and ng L^{-1}), leading to higher SMX degradation rates. Since acetate is a substrate routinely present in WWTPs, this suggests that the increased efficiency in SMX biodegradation at low concentrations may be achievable in real wastewater, while the higher growth rate of *A. denitrificans* PR1 as compared to e.g. nitrifiers and phosphorus accumulating organisms supports its potential to survive in WWTPs. Furthermore, the k_{bio} of the culture was 2 orders of magnitude higher than activated sludge even when acetate was not present as a supplemental substrate. Overall, these results suggest that the strain is a potentially interesting organism for bioaugmentation to achieve SMX removal. Further studies are required to validate the effectiveness of bioaugmenting *A. denitrificans* PR1 in activated sludge when wastewater influent is supplied, as well as to assess the removal mechanisms in activated sludge.

2.6. Acknowledgement

This research was supported by MERMAID, an Initial Training Network funded by the People Programme (Marie Skłodowska-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607492, and by UCIBIO (FCT/MEC UID/Multi/04378/2013, POCI-01-0145-FEDER-007728).

3

BIOAUGMENTATION OF ACTIVATED SLUDGE WITH *ACHROMOBACTER DENITRIFICANS* STRAIN PR1 FOR ENHANCING THE BIOTRANSFORMATION OF SULFAMETHOXAZOLE BIO- AND ITS HUMAN CONJUGATES IN REAL REAL WASTEWATER: KINETIC TESTS AND MODELLING

Abstract

Achromobacter denitrificans PR1 has previously shown potential to degrade the antibiotic sulfamethoxazole, whereby sulfamethoxazole biotransformation was stimulated in the presence of biogenic substrates. This study examined the biotransformation kinetics of sulfamethoxazole and its two main conjugates, N_4-acetyl-SMX and SMX-N_1-Glucuronide, by activated sludge and activated sludge bioaugmented with *A. denitrificans* PR1. SMX biotransformation under both anoxic and aerobic conditions was tested, with and without the addition of acetate as growth substrate, to understand the range of applicable conditions for bioaugmentation purposes. Biological process models, such as the pseudo-first order kinetic and cometabolic models, were also applied and, following the estimation of kinetic parameters, could well describe data measured in bioaugmented and non-bioaugmented AS batch experiments under various test conditions. Experimental and modelling results suggest that (i) retransformation of the two conjugates to SMX in AS occurred under both aerobic and anoxic conditions, and (ii) biotransformation kinetics of SMX can vary significantly depending on redox conditions, e.g., SMX was biotransformed by AS only under aerobic conditions. Notably, SMX biotransformation was significantly enhanced when PR1 was bioaugmented in AS. Addition of acetate as biogenic substrate is not neccessary, as PR1 was capable of enhancing the SMX biotransformation by using the carbon sources present in wastewater. Overall, bioaugmentation by means of *A. denitrificans* PR1 could be a viable strategy for enhancing SMX removal in AS wastewater treatment plants (WWTPs).

Keywords: antibiotics, cometabolism, N_4-acetyl-SMX, SMX-N_1-Glucuronide, modelling, retransformation

Published as:

Nguyen, P.Y., Carvalho, G., Polesel, F., Torresi, E., Rodrigues, A.M., Rodrigues, J.E., Cardoso, V.V., Benoliel, M.J., Plósz, B.G., Reis, M.A.M., Oehmen, A., 2018. Bioaugmentation of activated sludge with Achromobacter denitrificans PR1 for enhancing the biotransformation of sulfamethoxazole and its human conjugates in real wastewater: Kinetic tests and modelling. Chem. Eng. J. 352, 79–89. https://doi.org/10.1016/J.CEJ.2018.07.011

3.1. Introduction

The intensive use of antibiotics for human and veterinary therapy has led to their continuous discharge, also in the form of conjugates, in the environment. WWTPs are not designed to remove these and other xenobiotic chemicals, and discharge of treated effluents into the environment has been a major concern due to the risk of a worldwide dispersal of antibiotic resistance genes (Michael et al., 2013).

Amongst antibiotics, sulfamethoxazole (SMX) is one of the most widely used synthetic sulfonamides worldwide. SMX enters WWTPs via human excretion in the forms of unchanged SMX (15-25% of the excreted dose) as well as the conjugated forms N_4-acetyl-SMX (Ac-SMX) (> 40%) and SMX-N_1-Glucuronide (SMX-Glu) (9-15%) (Van der Ven et al., 1994). The two human conjugates have been detected in wastewater influent and effluent, and were observed to rapidly deconjugate during wastewater treatment (Göbel et al., 2007; Stadler et al., 2015) which was considered to likely explain the reported 'negative removal' of SMX in wastewater treatment (Plósz et al., 2010a; Stadler et al., 2015). This suggests the importance of investigating the retransformation of the two major human conjugates to parent SMX, in order to explain the reported differences in removal efficiencies in WWTPs (Joss et al., 2005; Plósz et al., 2010b).

SMX removal has been shown to vary greatly, i.e. from negative (-138%) to very high (>90%) (Michael et al., 2013) in full-scale WWTPs, and with variability in SMX biotransformation kinetics. SMX was also shown to be not readily biodegradable during the 28-day test period in a closed bottle test (Alexy et al., 2004).

Biotransformation has been recognized as the major elimination mechanism of SMX and its conjugates during biological treatment of domestic wastewater, with minor contribution of sorption onto sludge (due to the polar nature of these compounds). Overall, literature reports of inconsistent and incomplete SMX elimination suggest that novel technologies/strategies would be required if more stringent discharge limits for SMX and other antibiotics are enforced. Bioaugmentation can be an alternative WWTP operational strategy to enable or enhance xenobiotics removal by inoculating specialized degrading bacteria (Van Limbergen et al., 1998). Despite the fact that bioaugmentation has been studied for years in wastewater treatment to reinforce biological processes, few studies have tested the use of bioaugmentation for enhancing the removal of xenobiotics, e.g. 17β-estradiol (Roh and Chu, 2011), estradiol (Iasur-Kruh et al., 2011), fungicides (Wu et al., 2018). With respect to antibiotics, bioaugmentation resulted in limited SMX removal when applying *Microbacterium* sp. strain BR1 in full-scale membrane bioreactors (Fenu et al., 2015), except for SMX concentrations far higher than the ones normally found in municipal wastewater.

Previously, we showed that a pure culture of *Achromobacter denitrificans PR1* could exhibit faster biotransformation kinetics (up to two to three orders of magnitude higher) of SMX compared to AS alone (Nguyen et al., 2017), even at the low SMX concentrations typical of wastewater effluents. Given its ability to degrade SMX in the presence and/or absence of other additional carbon sources (acetate and succinate) at environmentally relevant concentrations (typical of e.g., wastewater effluents), the strain likely has potential for treating SMX in wastewater upon bioaugmentation. Therefore, the overall objective of this work was to investigate whether PR1 can enhance SMX biotransformation kinetics when bioaugmented to AS with real wastewater feed. Specifically, we (i) investigated the effect of redox conditions, i.e. aerobic and anoxic conditions, on the transformation rates of targeted compounds; (ii) assessed the potential influence of retransformation processes of the two main human conjugates, i.e. Ac-SMX and SMX-Glu, on the fate of sulfamethoxazole under the testing conditions; and (iii) evaluated the need for supplementation with a biogenic substrate (e.g. acetate) or whether the availability of carbon sources in wastewater could serve as biogenic substrates to achieve a sufficiently interesting kinetic for SMX removal upon bioaugmentation of AS with PR1. Modelling the fate of xenobiotics in WWTPs can be a useful tool to understand their removal mechanisms, predict and reduce their emissions with treated effluent through process optimization. Specifically, the Activated Sludge Modelling framework for Xenobiotics (ASM-X), has been previously used to predict the fate of SMX in biological treatment systems (Plósz et al., 2010b) and to identify factors (influent concentration of conjugates, solid residence time) possibly explaining the variability in SMX removal efficiencies (Polesel et al., 2016). In this context, suitable mathematical models were developed to examine the metabolic mechanism and predict kinetics of SMX and human conjugates biotransformation upon bioaugmentation of *A. denitrificans* PR1 into AS.

3.2. Materials and methods

3.3.1. Laboratory lab-scale experiments - Bioaugmentation of AS for enhancing SMX biotransformation

3.2.1.1. Culture media

Bacterial inoculum was grown in mineral medium B, supplemented with ammonium phosphate at concentration of 400 mM (designated as MMBN), as previously described by (Reis et al., 2014). The cells were harvested by centrifugation (7000 x g for 10 min at 20°C using a Sigma® 4-16KS centrifuge), and rinsed three times with fresh MMBN medium to remove the trace amount of SMX remaining from the culture medium before augmenting to the reactors to get an initial cell suspension concentration of approximately 0.05-0.06 $mg_{biomass}$ L^{-1}.

3.2.1.2. Batch tests

Biotransformation of SMX and the two main human conjugates by AS and bioaugmented AS was assessed in a series of batch experiments in 1 L jacketed glass reactors. Dried compressed atmospheric air or pure nitrogen were continuously sparged by a diffuser placed at the bottom of each reactor to create aerobic or anoxic conditions, respectively. Temperature was controlled at 20°C using an external recirculating bath and pH was monitored and maintained between 7.0-7.4 by the addition of HCl (0.2 M) or NaOH (0.2 M), using pH controllers (HI8711, Hanna Instruments, US) with dual set point.

For all experiments, primary effluent wastewater and AS (from a Modified Ludzack Ettinger system) collected from the Chelas WWTP (Lisbon, Portugal) were used. More information about Chelas WWTP is provided in Appendix B3 and Table B3. AS and primary effluent were seeded to the 1 L glass reactors at an initial biomass concentration of approximately 3 gTSS L^{-1} for all the experiments. Overall, four types of batch tests were performed: (i) abiotic control tests; (ii) sorption tests; (iii) bioaugmenation tests; (iv) nitrification inhibtion tests. The testing conditions are presented in **Error! Reference source not found.**. All the tests were performed in duplicate, except for the anoxic bioaugmented AS test (An2, Table 3.1), the control 1 and the allylthiourea (ATU) nitrification inhibition tests.

Abiotic control test (control 1)

The goal of this experiment was to determine the contribution of abiotic removal mechanisms (stripping, sorption onto reactor walls and equipment, and abiotic chemical reactions). In this test, the 1 L-glass-reactor was filled with Milli-Q water that was spiked with the three compounds, e.g. SMX, Ac-SMX and SMX-Glu at the concentrations of 10 µg L^{-1}, 15 µg L^{-1} and 15 µg L^{-1}, respectively. The experiment lasted 6 hours.

Sorption tests (control 2)

Sodium azide (NaN_3) is a well know respiration inhibitor used for negative control tests in AS studies. A wide range of concentrations from 0.5 to 720 $mg_{azide}g_{TSS}^{-1}$ were used in previous studies for this purpose (Hamon et al., 2014). In this test, a concentration of ~ 650 $mg_{azide}g_{TSS}^{-1}$ was used to inhibit AS activity. SMX, Ac-SMX and SMX-Glu were spiked into the reactors at the initial concentrations of 5 µg.L^{-1}, 10 µg.L^{-1} and 10 µg.L^{-1}, respectively. The tests were performed in duplicate.

Bioaugmentation tests

The goal of these tests was to assess biotranformation of the targeted compounds with non-bioaugmented and bioaugmented AS with *A. denitrificans* PR1. Batch experiments were performed

during 12 to 14 hours under aerobic and anoxic conditions. In aerobic tests, the influence of a biogenic substrate on SMX biotransformation was assessed by adding acetate at an initial concentration of ~ 137 to 152 $mg_{COD}L^{-1}$ that is similar to the level of soluble COD typically found in many activated sludge WWTPs.

In anoxic batch tests, reactors were supplemented with an initial nitrate concentration of 80 mg NO_3-N L^{-1} in the form of KNO_3. Aqueous stock solutions of SMX and the two target conjugates were spiked to obtain an initial concentration of approximately 5 µg L^{-1} and 10 µg L^{-1}, respectively.

Table 3.1. Overview of the different testing conditions of bioaugmented and non-bioaugmented AS

		Batch	**Feed**	**AS** (gTSS L^{-1})	***A.denitrificans* PR1** ($g_{biomass}$ L^{-1})	**Acetate** ($mg_{COD}L^{-}$)	**NaN_3** ($mg_{azide}g_{TSS}^{-1}$)	**ATU** (mg L^{-1})
Control	Control 1	C1	MilliQ-water	-	-	-		
	Control 2	C2	WW*	~ 3	-	-	~ 650	
Nitrification-inhibition		ATU	WW*	~ 3	-	-		30
		Without ATU	WW*	~ 3	-	-	-	-
Bioaugmen-tation tests	Aerobic	A1	WW*	~ 3	0	0		
		A2	WW*	~ 3	0	137-152		
		A3	WW*	~ 3	~ 0.05-0.06	0		
		A4	WW*	~ 3	~ 0.05-0.06	137-152		
	Anoxic	An1	WW*	~ 3	0	0		
		An2	WW*	~ 3	~ 0.05-0.06	0		

WW*: wastewater from the effluent of a primary sedimentation tank was centrifuged at 10000 x g for 15 min at 4ºC, and then filtered through Whatman® Glass microfiber filters, pore size 1.2 µm binder free, Grade GF/C before feeding to the reactors.

Nitrification inhibition tests

To determine the contribution of ammonia oxidizing bacteria and heterotrophs to the SMX bio-transformation in AS communities, biomass was inactivated using ATU at 30 mg L^{-1} (Park et al., 2017), a copper chelator that depletes copper ions from the active center of ammonia monooxygenases (AMO), therefore inhibiting ammonia oxidizing activity.

3.3.2. Chemicals and reagents

Reagent grade (purity ≥ 99%) SMX was purchased from Sigma-Aldrich. Ac-SMX, SMX-Glu and isotopically labelled Ac-SMX-d4, SMX-d4-Glu, SMX-d4 were obtained from Toronto Research

Chemicals, Inc. (TRC, Canada). Individual stock standard solutions were prepared on a weight basis in methanol and stored at -20°C. HPLC-grade methanol was supplied by Merck (Darmstadt, Germany).

3.3.3. Sample preparation and analytical procedures

Samples collected along the tests were centrifuged for 5 min at 8000 xg, followed by syringe filtration through 0.2 µm cellulose Whatman filters and stored at -20°C prior to analysis of soluble chemicals.

The acetate concentrations were determined by high-performance liquid chromatography (HPLC) using an IR detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.6 mL/min and a 50°C operating temperature.

Total and volatile suspended solids (TSS, VSS) were determined according to Standard Methods (APHA, 1995). Ammonium, nitrate and nitrite concentrations were measured using a segmented flow analyzer through the Skalar San++ system. Samples were also analyzied for soluble COD (sCOD) using HACH-lange test kits and a DR2800 spectrophotometer (HACH, Germany).

Analysis of SMX and the two human conjugates was performed on a high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. Detailed descriptions of the sample preparation and analytical methods used are provided as Appendix B2.

3.3.4. Determination of kinetic parameters-modelling approach

3.2.4.1. Modelling assumptions

In this study, we hypothesized that (i) only retransformation of the two conjugates, e.g. Ac-SMXl and SMX-Glu, will occur through deconjugation to form the parent compound SMX and that (ii) the dissolved compounds are the only biodegradable fractions. Thus, the biotransformation of SMX includes two processes: (i) formation of SMX due to the retransformation (deconjugation) of Ac-SMX and SMX-Glu; (ii) simultaneous elimination of SMX.

3.2.4.2. Model implementation and estimation of parameters

In this study, the biotransformation rate of the three target compounds was calibrated using the ASM-X modelling framework (Plósz et al., 2012, 2010b; Polesel et al., 2016).

Deconjugation of the two human conjugates (Ac-SMX and SMX-Glu) to form the parent compound SMX is described by a pseudo-first order kinetic model (Table 3.2, process (1) for aerobic and process (9) for anoxic removal), thus allowing the estimation of the biotransformation rate coefficients, e.g. $k_{Dec,Ox}$ or $k_{Dec,Ax}$ (L $gTSS^{-1}$ d^{-1}).

For the biotransformation of SMX under aerobic conditions, both pseudo-first order and cometabolic models were implemented to test which one could appropriately predict SMX biotransformation (Table 3.2). The cometabolic biotransformation model (Plósz et al., 2012) consisted of two biotransformation rates: the enhanced rate in the presence (q_{bio}, L d^{-1} g^{-1}) and the pseudo-first order rate in the absence (k_{bio}, L d^{-1} g^{-1}) of growth substrates. Accordingly, biotransformation kinetics of the cometabolic substrate (e.g. micropollutants) depend on the readily biodegradable growth substrates, S_S (mgCOD L^{-1}). S_S was determined as the difference between soluble COD (sCOD, measured during the experiments) and soluble inert COD (S_I – calculated according to (Roeleveld and Van Loosdrecht, 2002)). The initial S_S concentration of the pre-clarified municipal wastewater used in this study ranged between 41 and 128 mgCOD L^{-1}. Parameters that could not be identified through model calibration to experimental results (i.e. heterotrophic yields Y_H, substrate affinity constant K_S) were adopted from literature (Henze et al., 2000). Concentration profiles of acetate, expressed as sCOD, were used to calibrate the maximum specific growth rate of heterotrophs μ_H (Table B5 and Figure B2, Appendix B). The estimated parameters included: (i) biotransformation rate constants of the AS ($k_{bio,AS}$) and the bioaugmented strain ($k_{bio,PR1}$) in the absence of primary substrate; and (ii) the cometabolic biotransformation rate constants of the AS ($q_{bio,AS}$) and the bioaugmented strain ($q_{bio,PR1}$) in the presence of the primary substrates. Each batch test was designed to determine a specific kinetic constant and is described in Table 3.3. The model was implemented in Aquasim 2.1d (Reichert, 1994) and the embedded secant method was used for parameter estimation.

In our study, experimental data from A1, A4, An1 and An2 tests were used for the model calibration and estimation of the biotranformation rate constants of SMX and the two human conjugates by AS and *A. denitrificans* PR1 under aerobic and anoxic conditions (Table 3.3).

For model calibration, the $k_{bio,AS}$ value was approximated based on process (2) (Table 3.2) using the tangent value of the linear regression line fitted to measured data obtained in the primary substrate limitation period (from 6 hours to 14 hours, after retransformation was completed and growth substrates were depleted (Figure 2a). K_D values (shown in Table 3.2) were used to assess the sorption fraction, while the constant value X_{AS} in Table 2 represent activated sludge (AS) biomass concentration. Process (1), (4) and (6) (Table 3.2) allows estimation of $q_{bio,AS}$, using the $k_{bio,AS}$ value obtained above, the K_D value (shown in Table 3.2), and a constant value for X_{AS}.

Data obtained from the bioaugmented test A4 was used to determine the cometabolic biotransformation rate constant of *A. denitrificans* PR1, i.e. $q_{bio,PR1}$. Biotransformation of SMX in this experiment was attributed to the activity of both AS and the strain PR1 (Table 3.2, process (1), (4), (5) and (6)) and characterized by the biotransformation rate constants of AS ($k_{bio,AS}$ and $q_{bio,AS}$) and of PR1 ($k_{bio,PR1}$ and $q_{bio,PR1}$). Biotransformation kinetic associated with AS were previously estimated through

calibration against A1 test results, while $k_{bio,PR1}$ was derived from our previous study under primary substrate limitation (Nguyen et al., 2017).

The biotransformation of SMX under anoxic conditions is predicted using pseudo-first order kinetics, thereby allowing for the estimation of the biotransformation rates $k_{bio,Ax}$ (L gSS^{-1} d^{-1}).

Experimental results from An1 were used to determine the retransformation rate, i.e. $k_{Dec,Ax}$ and SMX biotransformation kinetics of AS, i.e. $k_{bio,AS}$, under anoxic conditions using processes (7) and (8) (Table 3.2). The anoxic $k_{bio,AS}$ was then used as an input for predicting An2 results and estimating the biotransformation rate coefficient of strain PR1 ($k_{bio,PR1}$) using processes (7), (8) and (9) (Table 3.2).

3.2.4.3. Model validation

Two different sets of experimental results (A2 and A3) were used to validate the cometabolic kinetic models calibrated with the data sets of A1 and A4.

Table 3.2. Stoichiometric (Gujer) matrix of the ASM-X for processes of parent compound retransformation, biotransformation and the alternative cometabolic biotransformation model. Parameters and state variables for determination of MPs kinetics are described in the main text.

Processes i → **j process ↓**	C_{LI}	C_{CJ}	S_s	X_{AS}	X_{PR1}*	**Process rate**
Pseudo-first order kinetics – Aerobic processes						
(1) *Parent compound formation due to retransformation of human conjugates* C_{CJ}	F	-1		*		$\frac{k_{Dec} C_{CJ} X_{AS}}{1+K_D X_{AS}}$
(2) Pseudo-first order kinetics – biotransformation transformation of parent compound C_{LI} *by AS*	-1			*		$\frac{k_{bio\_AS} C_{LI} X_{AS}}{1+K_D X_{AS}}$
(3) Pseudo-first order kinetics – biotransformation of parent compound C_{LI} *by the bioaugmentation strain, i.e.* A. denitrificans *PR1*	-1				*	$\frac{k_{bio\_PR1} C_{LI} X_{PR1}}{1+K_D X_{PR1}}$
Cometabolic model – Aerobic processes						
4) Cometabolic biotransformation of C_{LI} *by AS*	-1			*		$\frac{(q_{bio\_AS} \frac{S_S}{S_S+K_S} + k_{bio\_AS}) C_{LI} X_{AS}}{1+K_D X_{AS}}$
(5) Cometabolic enhancement biotransformation of C_{LI} *by the bio-augmentation strain, i.e.* A. denitrificans *strain PR1*					*	$\frac{(q_{bio\_PR1} \frac{S_S}{S_S+K_S} + k_{bio\_PR1}) C_{LI} X_{PR1}}{1+K_D X_{AS}}$
(6) Aerobic growth				*	*	$\mu_H \frac{S_S}{S_S+K_S} X_H$

Pseudo first order kinetics – Anoxic processes				
(7) Parent compound formation due to retransformation of human conjugates C_{CJ}	F	-1	*	$\frac{k_{Dec} C_{CJ} X_{AS}}{1 + K_D X_{AS}}$
(8) Biotransformation of parent compound C_{LI} by AS	-1		*	$\frac{k_{bio\_AS} C_{LI} X_{AS}}{1 + K_D X_{AS}}$
(9) Biotransformation of parent compound C_{LI} by the bio-augmentation strain, i.e. A. denitrificans *PR1*	-1		*	$\frac{k_{bio\_PR1} C_{LI} X_{PR1}}{1 + K_D X_{PR1}}$

*Due to short duration of the batch experiment and low S/X ratio, negligible biomass growth was assumed.

F = ratio between molecular mass of parent compound and metabolite undergoing deconjugation.

S_S: primary substrate concentration (e.g., organic matter or acetate in some of these experiments, expressed as readily soluble biodegradable COD) considering a co-substrate (gCOD L^{-1}).

C_{LI} and C_{CJ} : the aqueous concentrations of the parent compound and the human conjugates undergoing deconjugation to the parent compound, respectively (µg L^{-1}).

k_{Dec}: retransformation rate constant of deconjugation of the human conjugates to parent compound (L $gTSS^{-1}$ d^{-1}).

k_{bio_AS} : is the reaction rate coefficient of biotransformation of parent compound (L $gTSS^{-1}$ d^{-1}) by AS.

k_{bio_PR1}: is the reaction rate coefficient of biotransformation of parent compound (L $gTSS^{-1}$ d^{-1}) by the bioaugmented *A. denitrificans* strain PR1.

K_S : half-saturation coefficient for S_S

K_D: sorption coefficient (0.256 L $gbiomass^{-1}$ for SMX - (Göbel et al., 2005)). The values are not available for N4-acetyl-SMX and SMX-N_1-Glucuronide, and were thefore assumed to be equal to 0.

X_{PR1} or X_{AS} : biomass concentration of bio-augmented strain *A. denitrificans* or AS, expressed in gTSS L^{-1};

X_H is expressed in gCOD L^{-1} by assuming biomass-to-COD ratio of 0.75

Table 3.3. Model calibration and parameter estimation procedures for the batch tests performed under aerobic condition

Batch	Goal	Process used	Input parameters	Estimated parameters
A1 (non-bioaugmented)	Model calibration	Process (1) Process (2) Process (1), (4) and (6)	$k_{\_dec\_N4\_Ox}$, $k_{\_dec\_Glu\_Ox}$, $k_{\_bio\_AS\_Ox}$	$k_{\_dec\_N4\_Ox}$, $k_{\_dec\_Glu\_Ox}$ $k_{\_bio\_AS\_Ox}$ $q_{\_bio\_AS\_Ox}$
A4 (bioaugmented)	Model calibration	Process (3) Process (1), (4), (5) and (6)	$k_{\_dec\_N4\_Ox}$, $k_{\_dec\_Glu\_Ox}$, $k_{\_bio\_AS\_Ox}$, $q_{\_bio\_AS\_Ox}$, $k_{\_bio\_PR1\_Ox}$	$k_{\_bio\_PR1\_Ox}$[**] $q_{\_bio\_PR1\_Ox}$
A2 (non-bioaugmented)	Model validation	Process (1), (4) and (6)	$k_{\_dec\_N4\_Ox}$, $k_{\_dec\_Glu\_Ox}$, $k_{\_bio\_AS\_Ox}$, $q_{\_bio\_AS\_Ox}$	None
A3 (bioaugmented)	Model validation	Process (1), (4), (5) and (6)	$k_{\_dec\_N4\_Ox}$, $k_{\_dec\_Glu\_Ox}$, $k_{\_bio\_AS\_Ox}$, $q_{\_bio\_AS\_Ox}$, $k_{\_bio\_PR1\_Ox}$, $q_{\_bio\_PR1\_Ox}$	None
An1 (non-bioaugmented)	Model calibration	Process (7) Process (8)	$k_{\_dec\_N4\_Ax}$, $k_{\_dec\_Glu\_Ax}$	$k_{\_dec\_N4\_Ax}$, $k_{\_dec\_Glu\_Ax}$ $k_{\_bio\_AS\_Ax}$
An2 (bioaugmented)	Model calibration	Process (7), (8) and (9)	$k_{\_dec\_N4\_Ax}$, $k_{\_dec\_Glu\_Ax}$, $k_{\_bio\_AS\_Ax}$	$k_{\_bio\_PR1\_Ax}$

[**] $k_{\_bio\_PR1\_Ox}$ was determined in our previous study (Nguyen et al., 2017), from the test with the pure culture (i.e. *A.denitrificans* PR1 biodegradation test) conducted in mineral medium supplemented with SMX as the only substrate.

3.3. Results and discussions

3.3.1. Abiotic and sorption processes

Figure 3.1 shows the evolution of SMX, Ac-SMX and SMX-Glu concentrations over the test period of 6 h in control test 1, revealing 1.8%, 11.4% and 11.8% removal for Ac-SMX, SMX-Glu and SMX, respectively. This suggests that abiotic processes had minor contribution to the removal of the tested compounds, in agreement with previous studies (Li and Zhang, 2010).

To investigate sorption to AS, sodium azide (NaN_3) was used to inhibit the aerobic respiration and suppress the microbial activity of the AS (control 2). The results showed that Ac-SMX and SMX-Glu were transformed concomitantly with an increase in SMX concentration (Figure 3.1), indicating that the retransformation of the parent SMX from the two human conjugates occurred even with inactivated biomass, likely via extracellular enzymes. This is in agreement with previous studies for other conjugates (Ramin et al., 2016). In terms of mass balance, supposing that all the human conjugates were converted back to SMX, there was approx. 0.02 µmol SMX formed after 4.5 hours, while there was a removal of approx. 0.02 µmol of the two human conjugates. Therefore, no SMX removal was observed in the presence of NaN_3 (control 2). Since sodium azide was present at concentrations previously observed to be sufficient to inhibit the fraction of aerobic biomass (Barbot et al., 2010), biotransformation of the two human conjugates could be due to the activity of facultative anaerobic bacteria, which was not sufficiently inhibited by the addition of NaN_3.

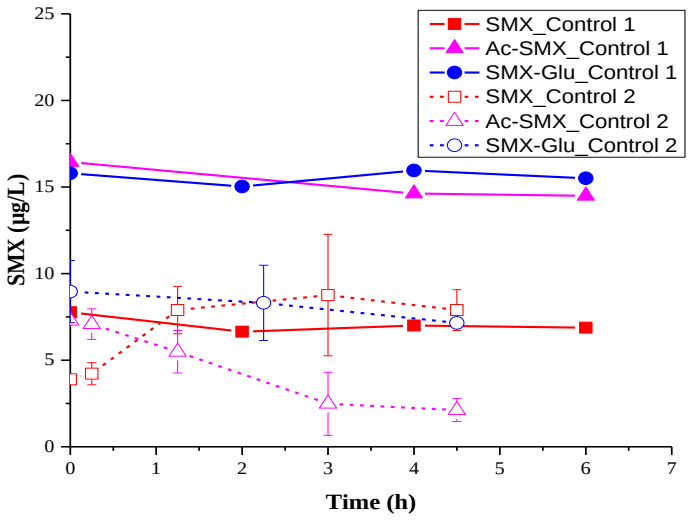


Figure 3.1. Measured concentrations of SMX, Ac-SMX, and SMX-Glu as a function of time for the control batch tests, i.e. control 1-with Milli-Q water (continuous lines), control 2-with NaN3 (dashed lines) as an inhibitor. Error bars indicate the standard deviations for duplicates

Due to the impossibility of determining the partitioning coefficient for SMX, the sorption fraction was assessed considering the sorption coefficient K_D obtained from previous literature. A K_D val-

ue of 0.256 L $gTSS^{-1}$ (Göbel et al., 2005) was chosen as the tests in this study were performed with fresh AS and real wastewater, which was representative of the real WWTPs where the K_D was obtained. At circumneutral pH typical of activated sludge systems, SMX is predominantly speciated as an anion (pK_a = 5.7). Possibly due to repulsion with negatively charged slugde particles, sorption of SMX has been generally found to be limited (K_d < 0.4 L g^{-1}) but not negligible. Notably, the K_d value used is in agreement with other determinations in activated sludge (see, e.g. (Plósz et al., 2010b), (Abegglen et al., 2009), (Göbel et al., 2005)). Sorption of the two human conjugates onto AS was not considered in these experiments as no reference values of sorption coefficient were reported. Indeed, the pH of the mixed liquor in the tests was between 7.0-7.4, which is well above the pK_a of SMX-Glu and Ac-SMX (pK_{a2} = 2.7 and 5.6, respectively). Under these experimental conditions, Ac-SMX and SMX-Glu exist predominantly or completely as negatively charged species in the aqueous phase. Hence, negligible sorption of Ac-SMX and SMX-Glu was assumed due to their high solubility and polar nature. In this case, k_{dec} can be considered as a generalized rate constant.

3.3.2. Biotransformation of SMX, SMX-Glu and Ac-SMX in bioaugmented and non-bioaugmented AS tests

In order to investigate the SMX transformation upon bioaugmentation of PR1 and the SMX retransformation in AS, several batch tests were performed: (i) A1 – only AS, aerobic conditons; (A2) – AS with supplementation of acetate under aerobic conditions; (A3) – AS bioaugmented with *A.denitrificans* strain PR1, under aerobic conditions; (A4) – AS bioaugmented with *A.denitrificans* strain PR1 with addition of acetate as a biogenic substrate to enhance SMX biotransformation by PR1 under aerobic conditions; (An1) – only AS under anoxic conditions; (An2) – AS bioaugmented with *A.denitrificans* strain PR1, under anoxic conditions; and two additional tests, with and without allythiourea (ATU) as an inhibitor to determine the contribution of ammonia oxidizing bacteria

3.3.2.1. Aerobic batch experiments (A1-A4)

In general, biotransformation of the two human conjugates was almost complete for all the batch tests performed under aerobic conditions. This is in agreement with previous studies, showing >85% removal of the two conjugates in laboratory-scale (Stadler et al., 2015) and full-scale (Göbel et al., 2007) AS processes, or even fully eliminated in a pilot membrane bioreactor (Mamo et al., 2018). In the aerobic non-bioaugmented reactors (A1, A2), an increase in SMX concentration was observed in the first 4–6 hours followed by a slow decrease during the rest of the tests when biotransformation of the two human conjugates was complete (Figures 3.2a and 3.2b). Negative SMX removal was observed, i.e. -43.1% and -63.8%, for the AS in tests A1 and A2, respectively. The decrease in Ac-SMX and SMX-Glu concentrations corresponded to increases in SMX concentrations (Figures 3.2a and 3.2b), strongly suggesting that the two human conjugates deconjugated rapidly to form the parent

compound SMX under aerobic conditions. There is relatively limited knowledge on the environmental fate and behavior of the conjugated pharmaceuticals, but these conjugates can undergo deconjugation reactions where deconjugation enzymes are present, with cleavage of the conjugated moiety, resulting in the formation of the parent pharmaceuticals (Kumar et al., 2012; Polesel et al., 2016).

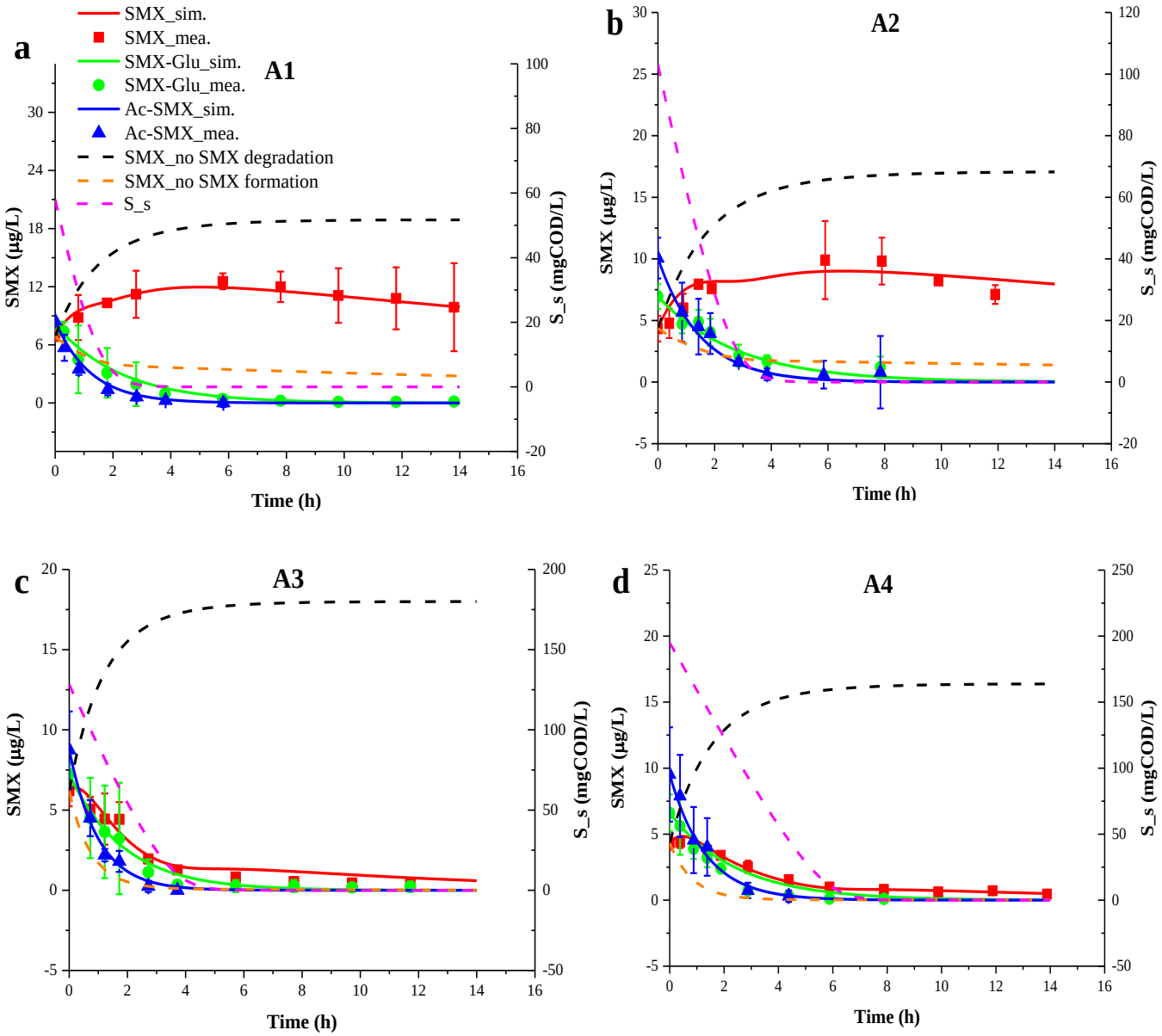


Figure 3.2. Illustration of measured concentrations of SMX, Ac-SMX, and SMX-Glu (markers) and simulated (lines) as a function of time for aerobic batch tests (A1): non-bioaugmented AS test (2a); (A2): non-bioaugmented AS test with supplementation of acetate as additional C-source (2b); (A3): bioaugmented AS with *A. denitrificans* PR1 test (2c); and (A4): bioaugmented AS with *A. denitrificans* PR1 supplemented with acetate test (2d). Orange dashed lines represent the SMX simulation if no SMX formation from the retransformation of Ac-SMX, and SMX-Glu. Black dashed lines represent the SMX simulation when all of Ac-SMX and SMX-Glu are converted back to parent SMX, but no SMX biodegraded. Error bars indicate the standard deviations for duplicates.

For bioaugmentation of AS with PR1, experimental results obtained in the two batches A3 and A4, show a comparably high rate of SMX biotransformation in the first 4 hours, followed by a relatively lower SMX removal rate after the growth substrates were completed (Figures 3.2c and 3.2d). similar SMX removal was observed in A3 and A4 after 12 hours, i.e. 92.5 ± 1.0% and 89.4 ± 1.5%, respectively. Concomitantly, complete removal of the SMX-Glu and Ac-SMX in the first 4 hours of the test was observed, which supposedly was converted back to SMX (Figure 3.2c and 3.2d) as suggested by other studies (Polesel et al., 2016; Stadler et al., 2015).

Relative contribution of heterotrophs and ammonia oxidizing bacteria

Batch tests performed in the presence of ATU showed no removal of ammonium and no formation of nitrate (Figure 3.3b), suggesting nitrification was completely suppressed There was 42 % removal of SMX after 12 hours (Figure 3.3a). No appreciable differences were observed in the removal efficiency of SMX with and without ATU (Figure 3a), suggesting negligible contribution of AOB to the biotransformation of SMX. Also, for all the remaining aerobic batch tests (A1, A2, A3, and A4) performed, no ammonium removal and no nitrate formation could be observed (Figure B3) during the testing periods, confirming that no nitrifying activity occurred in the tested AS. Even though biotransformation of SMX in AS was previously shown to correlate with both nitrifying activity (Kassotaki et al., 2016; Torresi et al., 2016) and heterotrophic bacteria (Alvarino et al., 2016; Müller et al., 2013), no appreciable differences were observed in the removal efficiency of SMX with and without ATU (Figure 3.3a) in our study, which could be due to the fact that (i) the SMX biotransformation rate by heterotrophic aerobic degradation was reported to be much faster compared to autotrophic nitrification (e.g. $k_{bio,\,h}$ = 0.09 L/g_{VSS} d vs. $k_{bio,\,a}$ = 0.01 L/g_{VSS} d) (Alvarino et al., 2016); and (ii) the possible higher abundance of heterotrophs compared to nitrifiers. Thus, heterotrophs seem to be the dominant organisms responsible for the biotransformation of SMX in the current study and the cometabolic model applied for all the batch tests could be based on only organic carbon (i.e. readily biodegradable substrates and supplemental acetate) as the primary substrates.

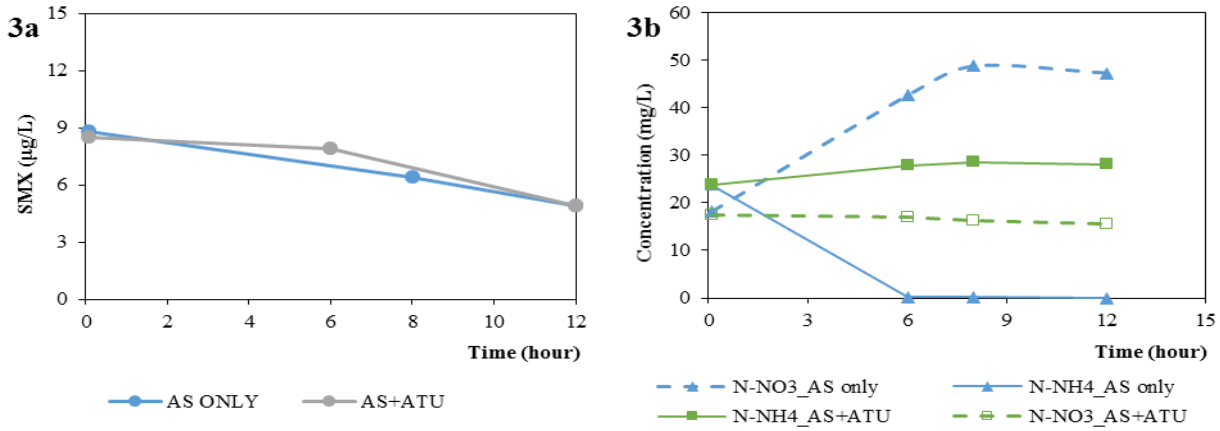


Figure 3.3. Effect of ATU inhibition on removal of SMX (3a) and nitrogen (3b)

Effect of acetate on the biotransformation of SMX

We previously showed that the presence of a biogenic substrate, e.g. acetate or succinate, provided for an 8-fold increase of SMX biotransformation kinetics by PR1 (Nguyen et al., 2017). In the current study, the initial concentration of acetate was approximately 137 to 152 mg COD L^{-1}, supplemented with preclarified wastewater to (i) the bioaugmented reactor (A4) (Figure 3.4b) as a biogenic substrate to enhance the kinetics of SMX biotransformation by PR1, and (ii) the non-bioaugmented reactor (A2) (Figure 3.4a) as a control for comparison purposes. Upon bioaugmentation of AS with PR1 (reactor A3, Figure 3.2c), with only wastewater (no acetate addition), SMX was biotransformed steadily and almost completely without any lag phase. In addition, the profiles of SMX in the tests fed with acetate were comparable to the one in the tests without (Figure 3.4a (A1 versus A2) and (Figure 3.4b (A3 versus A4)). From these observations, we hypothesized that (i) PR1 could use other available carbon sources present in wastewater as biogenic substrates to enhance the SMX biotransformation kinetics and the addition of acetate is unnecessary for the bioaugmentation with PR1; (ii) there is no enhancement effect due to acetate addition on the biotransformation of SMX by AS when fed with real wastewater. These hypotheses were also justified with the modelling results in section 3.3.3 and 3.3.4.

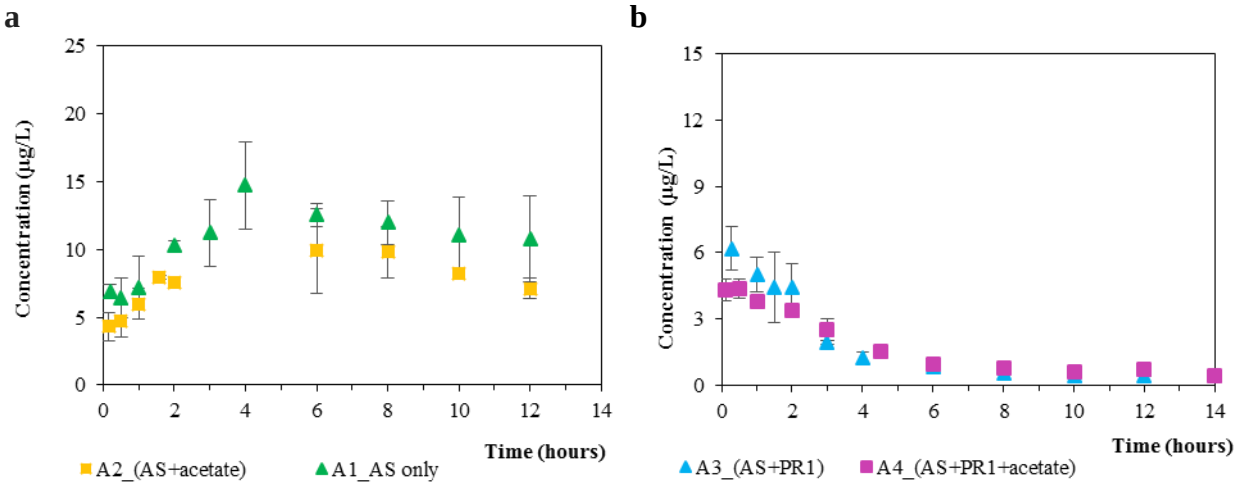


Figure 3.4. Measured concentrations of SMX as a function of time for the batch tests of non-bioaugmented AS (a) and bio-augmented one (b), with and without the addition of acetate under aerobic conditions. Error bars indicate the standard deviations for duplicates

3.3.2.2. Anoxic experiments

Pure culture biotransformation tests had previously shown that the *A.denitrificans* PR1 is capable of biotransformation of SMX under both aerobic (Nguyen et al., 2017) and anoxic conditions (data not shown). Hence the extent of SMX removal under anoxic conditions was also assessed and compared to those obtained in aerobic conditions.

In the two anoxic batch tests with non-bioaugmented (An1) and bioaugmented AS (An2), most of the two human conjugates were removed in the first 6 hours (Figure 3.5a and 3.5b), which is in

agreement with Stadler et al. 2015 (Stadler et al., 2015) that observed >90% of Ac-SMX and SMX-Glu removals under anoxic condition.

For the non-bioaugmented reactor An1, the consumption of nitrate (Figure 3.5c) revealed denitrifying activity, while no net SMX removal could be observed (Figure 3.5a) overall. SMX concentration increased in the first 6 hours simultaneously with the deconjugation of the two human conjugates, and remained constant for the rest of the experiment. This is opposed to what was observed in denitrifying AS (Plósz et al., 2010b) and denitrifying MBBR sludge (Polesel et al., 2017; Torresi et al., 2017). In contrast, in the bioaugmented reactor (An2), a slight decrease in SMX concentration was observed after the retransformations of the two human conjugates had completed (after 4 hours) (Figure 3.5b). This suggests a biotransformation of SMX associated with the activity of the bioaugmented strain PR1, but at a rather slow rate.

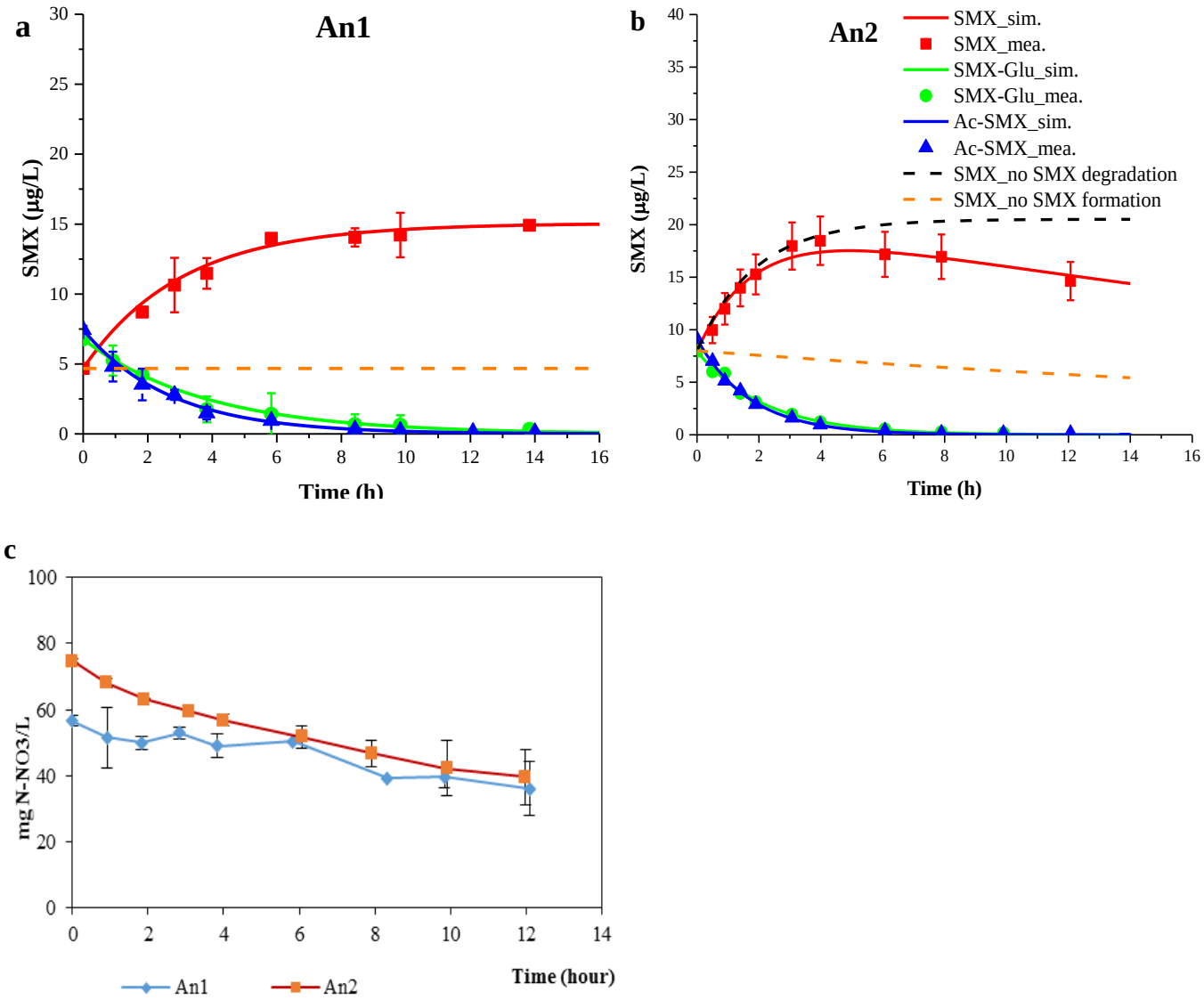


Figure 3.5. Illustration of measured concentrations of SMX, Ac-acetyl-SMX, and SMX-Glu (markers) and simulated (lines) as a function of time and nitrate consumption for the batch tests under anoxic conditions (An1): non-bioaugmented AS (4a); (An2): bioaugmented AS with *A. denitrificans* strain PR1 (4b). Orange dashed lines represent the SMX simulation if no SMX is formed from the retransformation of Ac-SMX, and SMX-Glu. Black dashed lines represent the SMX simulation when all of the Ac-SMX and SMX-Glu are converted back to SMX, but no SMX is biodegraded. Error bars indicate the standard deviations for duplicates.

3.3.3. Model-based assessment of biodegradation kinetics

Experimental data obtained in the batches A1, A4 and An1, An2 was used for the estimation of the biotransformation rate constants for AS and the *A. denitrificans* PR1 under aerobic and anoxic conditions. Predicted dissolved concentration profiles of SMX and its two human conjugates during batch experiments are compared with measured data and shown in Figures 3.2a, 3.2d and Figures 3.5a-b. The estimated parameters are summarized in Table 3.4. The model predictions were evaluated using the R-squared (R^2) coefficient, shown in Figures B4-B6, and summarized in Table B4 (Appen-

dix B). Confidence intervals were obtained by the estimation of standard deviations for a level of confidence of 95% (Figures B4-B6, Appendix B).

3.3.3.1. Kinetics of deconjugation of the two human metabolites

Deconjugation kinetics of the two conjugates could be described with pseudo-first order kinetics, in processes for aerobic (1) and anoxic (9) conditions. For the conjugated Ac-SMX, fitting of measured data resulted in k_{Dec} values of 8.9 ± 0.53 L $gTSS^{-1}$ d^{-1} for aerobic conditions, which was almost 2-fold higher than anoxic conditions (5.30 ± 0.21 L $gTSS^{-1}$ d^{-1}) (Table 3.4). These data agree well with values reported in literature for k_{Dec} of 5.9-7.6 L $gTSS^{-1}$ d^{-1} (Joss et al., 2006b; Plósz et al., 2010b) under aerobic conditions or 7.9 L $gTSS^{-1}$ d^{-1} (Plósz et al., 2010b) under anoxic conditions. No difference in the rate constants of SMX-Glu under aerobic and anoxic conditions was obtained (4.76 ± 0.4 and 4.74 ± 0.31 L $gTSS^{-1}$ d^{-1}, respectively). No data for the biotransformation rate coefficients of SMX-Glu were available in literature for comparison. Good agreement between experimental data and model simulations for Ac-SMX and SMX-Glu was shown, as confirmed by high R^2 coefficients (≥0.98), indicating that pseudo-first order equations describe well the biotransformation kinetics of the two human conjugates. These results suggest that (i) deconjugation rate constants are well above 1 L $gTSS^{-1}$ d^{-1}, thus indicating high degradability for conjugates; and (ii) deconjugation kinetics depend on redox conditions for Ac-SMX only, being faster under aerobic conditions.

3.3.3.2. Kinetics of SMX biotransforamtion under aerobic conditions

The removal of SMX under aerobic conditions was predicted using different mathematical models for comparision (i) a pseudo-first order kinetic model; and (ii) cometabolic models. Figure 3.2 and Figure B7 compare predicted and measured concentrations of SMX in the batches A1-A4, using a cometabolic model and a pseudo first order model, respectively. According to the data plotted in these figures, the prediction of SMX biotransformation was significantly improved by adopting the cometabolic model (R^2 ranged from 0.79 to 0.99, Table B4, Appendix B) compared to pseudo-first order biotransformation model (R^2 ranged from 0.044 to 0.94). These results: (i) show that the cometabolic model was able to consistently describe the experimental data, with measured concentrations that fall well within the 95% confidence interval (Figures B4, B5, Appendix B), making the cometabolic model the relevant choice for description of the removal of SMX; (ii) support our hypothesis of the deconjugation of Ac-SMX and SMX-Glu results in the formation of parent compound SMX, which likely explains the previously observed variability/negative SMX removal efficiencies in biological treatment. In this study, we also simulated two other scenarios for the fate of SMX, i.e.: (i) biotransformation of the two human conjugates leading to the formation of a compound different from SMX (model simulations presented as blue dashed lines in Figures 3.2 and 3.5); and (ii) all of the Ac-SMX and SMX-Glu are converted back to parent SMX, but no SMX is biodegraded, and the model simula-

tion is presented as black dashed lines in Figure 3.2 and Figure 3.5. However, the model simulations in these scenarios were far different from the respective observed concentrations of SMX in all the batch experiments (Figures 3.2 and 3.5), indicating that neither situation is applicable in the current study.

According to our simulation results, a SMX biotransformation rate constant $k_{bio,AS}$ of 0.47 ± 0.03 L $gTSS^{-1}$ d^{-1} and a cometabolic biotransformation rate constant q_{bio_AS} of 7.97 ± 0.51 L $gTSS^{-1}$ d^{-1} (Table 3.4) were obtained by AS under aerobic conditions. Previous studies reported $k_{bio,SMX}$ of 0.14 – 0.41 L $gTSS^{-1}$ d^{-1} (Abegglen et al., 2009; Alvarino et al., 2016; Min et al., 2018; Plósz et al., 2010b; Suárez et al., 2010), in agreement with our $k_{bio,AS}$ result.

For the bioaugmented AS, test A4 – with acetate supplementation, values of measured SMX concentration data plotted as a function of time elapsed show comparably high biotransformation rate in the first 4-6 hours (when primary substrate was available), followed by lower removal rate during the remaining time (following primary substrate depletion) (Figure 3.2d). By using the obtained parameters $k_{bio,AS}$, q_{bio_AS} from the A1 test, $k_{bio,PR1}$ (56.20 ± 3.70 L gTSS d^{-1}) from our previous study (Nguyen et al., 2017), fitting of measured data in the substrate depletion phase using cometabolic model as described in Table 3.2 resulted in estimation of $q_{bio,PR1}$ = 528.39 ± 6.78 L $gTSS^{-1}$ d^{-1}. This $q_{bio,PR1}$ value is consistent with rate constants ($k_{bio,PR1}$ = 445.6 – 570.1 L $gTSS^{-1}$ d^{-1}) previously obtained with PR1 when acetate was supplemented as a biogenic substrate to enhance the biotranforamtion rate of SMX in pure culture biodegradation tests (Nguyen et al., 2017). Higher biotransformation kinetics of SMX by PR1 ($q_{bio,\ PR1}$ and $k_{bio,\ PR1}$), compared to the retransformation kinetics of the two human conjugates (k_{Dec}), likely lead to the observation of no increase in SMX concentration in bioaugmented AS tests (A3 and A4), differently than what was observed in non-bioaugmented AS tests (A1 and A2).

In general, two different kinetic rates of theremoval of SMX are obtained for AS as well as for *A. denitrificans* PR1: a fast rate q_{bio} when primary substrate was available and a slower rate k_{bio} when primary substrate was depleted. These results can likely explain the two patterns of SMX biotransformation observed in bioaugmented batch tests A3 and A4 (Figures 3.2c and 3.2d). As a result of cometabolism, SMX removal was enhanced in the presence of primary substrates (as characterized by q_{bio}), with a subsequent decrease of biotransformation kinetics upon primary substrate limitation (characterized by the k_{bio}) at the end of the A3 and A4 tests (Figures 3.2c and 3.2d).

Also, the significant differences between k_{bio} and q_{bio} imply that growth substrates (readily biodegradable substrates) availability can substantially impact the removal of SMX as a result of cometabolism. In fact, typically present in wastewater at very low concentrations (ng L^{-1} to µg L^{-1}), micropollutants are unable to support cell replication and primary substrates (e.g. readily biodegradable carbon sources or ammonium) are essential for biomass growth and to induce enzymes for assimila-

tion or co-factors for biotransformation (Arp et al., 2001). As wastewater is a complex medium where not only micropollutants but also organic matter and nutrients are present, which could be degraded simultaneously by AS, cometabolism kinetics could be suitable to predict the behavior of micropollutants in real WWTPs.

In addition, the SMX biotransformation rate constants by *A. denitrificans* PR1, e.g. $q_{bio,PR1}$ in the presence and $k_{bio,PR1}$ in the absence of growth substrates, are three and two orders of magnitude higher, respectively, than that estimated for AS, confirming a specialized biotransformation capability by PR1 in comparison to the mixed AS community. Thus, bioaugmentation of AS with PR1 substantially enhanced the biotransformation rate of SMX.

3.3.3.3. Kinetics of SMX under anoxic conditions

SMX retransformation and removal under anoxic conditions can be predicted using pseudo-first order kinetics (processes (7) – (9)), thereby allowing for the estimation of $k_{bio,Ax}$ (L $gTSS^{-1}$ d^{-1}). In Figures 3.5a-b, simulated and corresponding measured concentrations of the three compounds in An1 and An2 batch experiments are plotted. High R^2 values (≥0.98) were obtained, and measured concentrations always fell within the 95% confidence interval (Figure B6, Appendix B), indicating that the pseudo-first order model was able to predict the fates of SMX and the two human conjugates obtained in the anoxic experiments. The estimated $k_{bio,Ax}$ for SMX biotransformation are 13.57 ± 2.10 and 0 L $gTSS^{-1}$ d^{-1} for *A. denitrificans* PR1 and AS, respectively (Table 3.4). The latter value is in contrast with other studies. Plósz et al. (2010) obtained a SMX biotransformation rate constant of 0.41 L $gTSS^{-1}$ d^{-1} under anoxic conditions with AS. In other studies, values of 0.1 and 0.05 L $gTSS^{-1}$ d^{-1} were reported for SMX biotransformation rate constants of heterotrophic denitrification and autotrophic denitrification, respectively (Alvarino et al., 2016). Torresi et al. (Torresi et al., 2017) reported a rate constant k_{bio} of 0.1 ± 0.1 L $gTSS^{-1}$ d^{-1} and q_{bio} of 1.7 and 3.2 for SMX biotransformation in a post-denitrification MBBR system dosed with methanol and ethanol, respectively. The obtained results suggest that, upon bioaugmentation to AS, PR1 could also be able to degrade SMX under anoxic conditions but at a significantly lower rate as compared to aerobic conditions – decreasing by 4-fold in terms of the rate constant ($k_{bio,PR1}$) under anoxic conditions as compared to aerobic conditions (Table 3.4).

In the current study, we also provided a detailed description of SMX removal in AS processes when assessing the biotransformation of the parent compound and the deconjugation of the two major human conjugates (Ac-SMX and SMX-Glu) back to SMX. SMX formation from the deconjugation of the two human conjugates was experimentally observed and comfirmed by model-based predictions. Ac-SMX and SMX-Glu were detected at levels that are comparable to the SMX concentrations in WWTPs (Joss et al., 2005; Wang and Gardinali, 2014) (see also Table B3, Appendix B). Significant

retransformation of SMX can take place in WWTPs at a higher rate compared to its removal rate (Table 3.4), resulting in the sometimes negative or varied SMX removal that have been observed in many studies. It implies that deconjugation of human conjugates should be taken into account to thoroughly understand the fate and removal of SMX during wastewater treatment.

In general, the results of these tests highlight the potential application of *A. denitrificans* PR1 for bioaugmentation for SMX removal in WWTPs. One criterion for a successful bioaugmentation is the metabolically active inoculum of a microorganism or consortium. Inability of the inoculated strains to degrade the xenobiotic chemicals once augmented into AS has been reported (McClure et al., 1991). One explanation given for such failure in bioaugmentation was the presence of alternative readily biodegradable substrates (McClure et al., 1991). In our experiments, enhancement of SMX biotransformation upon bioaugmentation of AS with PR1 was observed. Upon bioaugmentation of AS with *A. denitrificans*, without lag phase, a fast biotransformation of SMX was observed at rates similar to those obtained in pure culture biodegradation tests when acetate was supplemented as biogenic substrate. In addition, the SMX reaction rate constant and cometabolic biotransformation rate of PR1 were about two orders of magnitude higher than the kinetics of AS, regardless of the presence of additional acetate. The fact that the strain was able to use the complex substrates present in real wastewater to stimulate the activity and provide energy for growth and maintenance, suggests that PR1 has a great potential to survive in AS communities upon bioaugmentation. Overall, bioaugmentation with PR1 appears to be a feasible solution for enhancing SMX removal in wastewater, while further studies should focus on long-term biotransformation activity and stability of the bioaugmentation strain in wastewater systems in order to make bioaugmentation applicable.

Table 3.4. Model parameters and estimated kinetics for the biotransformations of SMX and the two human conjugates by activated sludge and *A. denitrificans* PR1 (PR1). Values in brackets indicate literature references.

Symbol	Definition	Unit	Compound					
			SMX-Glu		Ac-SMX		SMX	
			AS	PR1	AS	PR1	AS	PR1
			Aerobic					
$k_{Dec,Ox}$	Aerobic biotransformation rate coefficient for the human metabolites, C_{CJ}	L g X_{SS}^{-1} d^{-1}	4.76 ± 0.38 (n.a)	-	8.9 ± 0.53 (5.9-7.6[1]) (6.8[2])	-		
$k_{bio,Ox}$	Aerobic biotransformation rate coefficient for the parent compound, C_{LI}	L g X_{SS}^{-1} d^{-1}	-	-	-	-	0.47 ± 0.03 (0.19-0.2[3]) (0.3[4]) (0.41[2]) (< 0.1[5]) (0.1 ± 0.1[6])	56.20 ± 3.70
$q_{bio,Ox}$	Aerobic cometabolic-biotransformation rate constant for parent compound, C_{LI}	L g X_{SS}^{-1} d^{-1}	-	-	-	-	7.97 ± 0.51 (1.7 ± 0.2[6])	528.39 ± 6.78
			Anoxic					
$k_{Dec,Ax}$	Anoxic biotransformation rate coefficient for the human metabolites, C_{CJ}	L g X_{SS}^{-1} d^{-1}	4.74 ± 0.31 (n.a)	-	5.30 ± 0.21 (7.9[2])	-	-	-
$k_{bio,Ax}$	Anoxic biotransformation rate coefficient for the parent compound, C_{LI}	L g X_{SS}^{-1} d^{-1}	-	-	-	-	0	13.57 ± 2.10

[1](Joss et al. 2006b); [2](Plósz et al. 2010b); [3](Abegglen et al. 2009); [4](Suarez et al. 2010); [5](Joss et al. 2006b); [6](Torresi et al. 2017); n.a.: not available

3.3.4. Model validation

The models for retransformation of SMX from the two human conjugates and cometabolic biotransformation of SMX were validated using the two sets of experimental results, A2 and A3, for the non-bioaugmented and bioaugmented cases, respectively (Figures 3.2b and 3.2c). The set of estimated parameter values ($k_{Dec,N4}$, $k_{Dec,Glu}$, $k_{bio,AS}$, $q_{bio,AS}$, $k_{bio,PR1}$, $q_{bio,PR1}$) was used to test the capability of the proposed models to predict the behaviour of the three compounds (SMX, Ac-SMX and SMX-Glu) in the reactors A2 and A3, providing measured data independent from those used for model identification. Measured and predicted concentrations were compared and R^2 was calculated to determine the extent of correlation. Good agreement between the experimental data and model simulations could be observed with high R^2 ($\geq$0.95, Table B4). This indicates the applicability of the model towards the prediction of the fate of SMX and human conjugate biotransformation by both AS and PR1 (Figures 3.2b and 3.2d), even in the presence of an externally dosed carbon source (in this case acetate).

As cometabolic biotransformation depend on the readily biodegradable growth substrates, S_S (mgCOD L^{-1}), the addition of acetate to AS would affect the biotransformation of SMX. From our previous study (Nguyen et al., 2017) as well as the modelling result of the bioaugmentation test A4 (section 3.3.3.2), there is no doubt that acetate is a biogenic substrate to enhance the SMX biotransformation by *A. denitrificans* PR1, i.e. primary substrate for the cometabolism of SMX. For the non-bioaugmented activated sludge (A2), we hypothesized above (section 3.3.2.1.2) that there is no enhanced effects of acetate on the biotransformation of SMX by AS. To test this hypothesis, for modelling of SMX biotransformation of non-bioaugmented AS (batch A2 – with the supplementation of acetate), we tested, (i) both acetate and other readily biodegradable substrates that are present in wastewater (expressed as sCOD); (ii) only readily biodegradable substrates that are present in wastewater (expressed as sCOD) were considered as the primary substrates (S_S) in the cometabolic model to enhance the SMX biotransformation by AS. However, only the latter option gave good fitting between measured and model-based prediction (Figure B8, Appendix B vs. Figure 3.2b), suggesting that the readily biodegradable substrates that are present in wastewater (expressed as sCOD) acted and were sufficient as primary substrates for the cometabolism of SMX by AS. Acetate was measured in the wastewater and it was typically below 7 mg/L, therefore the microorganisms were probably not particularly adapted to it. Müller et al., (2013) observed that SMX cometabolism with acetate by AS occurred only after a sufficient adaptation time, meaning that the supplementation of additional acetate might have still enhanced the SMX further if sufficient adaptation time was allowed, although little is known about which easily biodegradable compounds are used as primary substrates by the AS community. Although the R^2 calculated for the SMX in the A2 test (Figure 3.2b) was equal to 0.79, the difference between measured and predicted SMX concentrations were still within the standard devia-

tions of the measured concentrations and falls in between confidence interval boundaries (Figure B3), making the cometabolic model term still relevant.

Model calibration and validation results revealed that the applied models could predict accurately the fate of SMX, Ac-SMX and SMX-Glu. Kinetic parameter values describing the biotransformation of SMX, Ac-SMX and SMX-Glu under aerobic and anoxic conditions could therefore be implemented in AS models linking organic carbon removal (heterotrophic activity) and xenobiotic biotransformation to predict the fate of SMX in WWTPs. Overall, modelling is fundamental to understand the kinetics and the contribution of different members in bioaugmented AS communities with respect to xenobiotic biodegradation. Thus, combining modeling and experimental data offers the opportunity for a thorough understanding of elimination mechanisms of micropollutants in WWTPs to facilitate optimization of wastewater treatment processes and reduce emissions of xenobiotics.

3.4. Conclusions

In this study, six different batch tests with non-bio-augmented and bio-augmented (with *A. denitrificans* PR1) AS, operated under different redox conditions, achieved different levels of SMX removal. Based on the experimental and model-based predictions, the following conclusions could be drawn:

- The biotransformation of SMX and deconjugation of Ac-SMX clearly depends on the redox conditions and the highest removal occurs under aerobic conditions.
- Based on experimental results and model-based observations in this study, we are able to confirm the conversion of the two conjugated human metabolites Ac-SMX and SMXGlu back into the parent compound SMX, which likely explains the previously observed variability/negative SMX removal efficiencies in biological treatment.
- Bioaugmentation of AS (AS) with *A. denitrificans* strain PR1 has led to superior biotransformation rates of SMX (by about two orders of magnitude) compared to the non-bioaugmented AS, within a complex carbon environment found at a WWTP without an addition of another C-source (acetate) as specific substrate for the biotransformation of sulfamethoxazole (SMX). These results prospect the use of *A. denitrificans* PR1 for bioaugmentation as a feasible and efficient option to improve SMX elimination in WWTPs.
- Cometabolic models and pseudo-first order kinetics were successfully calibrated to accurately predict the biological transformation kinetics of SMX and the two human metabolites, respectively, in both bioaugmented and non-bioaugmented reactors, under various redox conditions.
- The estimated kinetic parameters obtained from this study could be integrated in AS models to predict the fate of SMX during the biological treatment in WWTPs.

3.5. Acknowledgement

This research was supported by MERMAID, an Initial Training Network funded by the People Programme (Marie Skłodowska-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607492, and by UCIBIO (FCT/MEC UID/Multi/04378/2013, POCI-01-0145-FEDER-007728).

4

MEMBRANE BIOREACTOR BIOAUGMENTATION WITH *ACHROMOBACTER DENITRIFICANS* STRAIN PR1 FOR SULFAMETHOXAZOLE REMOVAL

Abstract

Achromobacter denitrificans strain PR1, previously found to harbor specific degradation pathways with high sulfamethoxazole (SMX) degradation rates, was bioaugmented into laboratory-scale membrane bioreactors (MBRs) operated under aerobic conditions to treat SMX-containing real domestic wastewater. Different hydraulic retention times (HRTs), which is related to reaction time and loading rates, were considered and found to affect the SMX removal efficiency. The availability of primary substrates was important in both bioaugmented and non-bioaugmented activated sludge (AS) for cometabolism of SMX. High HRT (24 h) resulted in low food to microorganism ratio (F/M) and low SMX removal, due to substrate limitation. Decrease in HRT from 24 h to 12 h, 6 h and finally 4 h led to gradual increases in primary substrates availability, e.g. organic compounds and ammonia, resulted in increased SMX removal efficiency and degradation rate, and is more favourable for high-rate wastewater treatment processes. After inoculation into the MBRs, the bioaugmentation strain was sustained in the reactor for a maximum of 31 days even though a significant decrease in abundance was observed. The bioaugmented MBRs showed enhanced SMX removal, especially under SMX shock loads compared to the control MBRs. The results of this study indicate that re-inoculation is required regularly after a period of time to maintain the removal efficiency of the target compound.

Keywords: membrane bioreactors, bioaugmentation, cometabolism, wastewater, antibiotics

Manuscript submitted as:

P.Y. Nguyen, Ana Filipa Silva, A.C. Reis, O.C. Nunes, Alexandre Mourão Rodrigues, João E. Rodrigues, Vitor Vale Cardoso, Maria João Araujo Gouveia Benoliel, M.A.M. Reis, A. Oehmen, G. Carvalho, "Bioaugmentation of membrane bioreactor with *Achromobacter denitrificans* strain PR1 for enhanced sulfamethoxazole removal in wastewater", Science of the Total Environment. Revised manuscript was submitted.

4.1. Introduction

SMX is an extensively prescribed or administered synthetic sulfonamide antibiotic to diminish inflammation and promote livestock growth (Zhang et al., 2016). Due to its incomplete metabolism and elimination, SMX is continuously released into the aquatic environment via wastewater treatment plants (WWTPs). The compound was categorized as Class 1: high priority pharmaceuticals relevant to the water cycle identified in a European assessment (de Voogt et al., 2009). Due to its polar and low adsorptive characteristics, SMX is mainly removed via biodegradation (Müller et al., 2013) as opposed to adsorption. There have been many studies on SMX elimination through biodegradation in lab-scales and full-scales. However, the results are widely varied, with removal efficiency ranging from negative to very high (>90%) (Müller et al., 2013). Inconsistent results hinder the optimization of the treatment process in WWTP systems. This fact, together with the ubiquitous presence of SMX in the environment, suggests that additional measures should be employed to enhance removal of SMX in poor performing WWTPs or in hospital/pharmaceutical wastewater treatments, especially if lower discharge levels of SMX are to be achieved to reduce the unwanted releases of antibiotics into the environment.

Bioaugmentation involves the addition of specialized microbial strains/consortia or genetically modified organisms to polluted hazardous waste sites or bioreactors in order to enable or hasten the biodegradation of targeted pollutants (Van Limbergen et al., 1998). In recent decades, bioaugmentation has been intensively studied to enhance removal of chlorinated pollutants (Santharam et al. 2011) in wastewater; phenolic compounds in wastewater (Fang et al. 2013); 3-chloroaniline (Boon et al., 2002); and other recalcitrant pollutants (Ma et al., 2009). However, wash-out of inoculated strains was reported to cause bioaugmentation failure (McClure et al., 1991), which could be overcome by using MBR. This combination of a biological process with membrane separation can potentially be a suitable substitute of traditional bioreactors for applying bioaugmentation as a means to retain all of the biomass and facilitate adaptation of the inoculated microorganism within the bio-system. Bioaugmentation of MBR has been successfully used to treat e.g. bromoamine acid wastewater (Qu et al., 2009), and atrazine containing wastewater (Liu et al., 2008). However, failure has been reported for bioaugmentation of MBR with a *Microbacterium* sp. strain BR1 for treatment of SMX in two pilot-scale MBR treating full-scale MBR effluent (post-treatment) or raw municipal wastewater (Fenu et al., 2015). The failure was attributed to the very low SMX concentrations, which could be limiting for the inoculated biomass, as well as the fact that lower SRT operational condition compared to the doubling time of *Microbacterium* sp. giving no chance for the survival of the strain in realistic application.

We previously proved that *A. denitrificans* PR1 is capable of using SMX as a carbon source (Reis et al., 2014) and is a promising organism for bioaugmentation to enhance SMX removal in wastewater due to its superior kinetics compared to AS, at environmentally relevant concentrations (Nguyen et al., 2017). This study aimed at the potential for bioaugmentation of MBR with *A. denitrifi-*

cans PR1 as an advancement strategy for improved SMX removal in WWTPs. An MBR was used as a strategy to hamper the release of the bioaugmentation strain into the environment. The influence of operational conditions, such as HRT, as well as necessity for amendment with a specific substrate (acetate) to augment the kinetics of PR1 upon bioaugmentation were investigated. Real pre-clarified effluent was used, not only to mimic the real conditions in WWTPs, but also to test for interactions between the inoculated strain and components of a complex medium, including organic carbon pollutants and ammonia. N_4-acetyl-sulfamethoxazole (Ac-SMX), one of the main human conjugated metabolites, previously observed to be reverted back to its parent compound SMX (Göbel et al., 2005) can interfere with the SMX removal efficiencies during WWTP. In this study, Ac-SMX was also monitored in the influent and effluent of the reactors to assess its transformation during treatment.

4.2. Materials and methods

4.3.1. Chemicals and reagents

Reagent grade (purity ≥ 99%) SMX, were purchased from Sigma-Aldrich. Ac-SMX and isotopically labelled Ac-SMX-d4 and SMX-d4 were obtained from Toronto Research Chemicals, Inc. (TRC, Canada). All other reagents were of analytical grade from commercial sources. Individual stock standard solutions were prepared on a weight basis in methanol and stored at -20ºC. A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions in Milli-Q water. HPLC-grade methanol and were supplied by Merck (Darmstadt, Germany).

4.3.2. Membrane bioreactors set up

Five different tests with bioaugmented (R1) and non-bioaugmented (R2) MBRs were conducted under continuous regime between 15 and 36 days, using pre-clarified effluent wastewater (as feed) and AS (as indigenous population) collected from a municipal WWTP, Chelas (Lisbon, Portugal) (see Appendix B3 for further information). For each test, two 1 L jacketed glass reactors coupled with side stream UFP-500-E-4MA (GE Healthcare, US) hollow-fiber membranes (surface area of 0.042 m^2, with a pore size of 500 000 NMWC (nominal molecular weight cut-off)) were operated in parallel, with initial sludge concentrations of approx. 2.8-3.5 gTSS L^{-1}. Fresh AS was used at the start of each conducted experiment. A diffuser was placed at the bottom of each reactor, and dry compressed atmospheric air was sparged continuously to create saturated aerobic condition (air flow of approximately 1 volume/volume/minute). Reactors were further equipped with an overhead stirrer, operated at a velocity of about 250 rpm. Temperature was controlled at 20°C and pH was maintained between 7.0-7.4 by the addition of HCl (0.05 M) or NaCl (0.05 M) using pH controllers (HI8711, Hanna Instruments, US) with dual set point.

The MBRs, named R1, were additionally bioaugmented with *A. denitrificans* PR1 (ranged from 1.1-4.2 % of TSS) to enhance the SMX degradation. The influent (feed) of MBRs was fortified with

SMX at a concentration of 10 µg L^{-1} (in M1, M2 and M3 tests) or 1 µg L^{-1} in M4 and M5 tests (Table 4.1), and dosed continuously to the MBRs using a peristaltic pump (Watson/Marlow Ltd., Cornwall, UK). Shock loads of 20 µg L^{-1} SMX were applied in some experiments at different time points (Table 4.1). The effluent (permeate) was pumped out of the reactors at the same flow rate than that of influent to maintain a constant reactor volume, using a peristaltic pump (Watson/Marlow Ltd., Cornwall, UK). Manual cleaning of the membranes was performed whenever necessary to prevent membrane clogging. After cleaning, the sludge cakes formed inside the membrane modules were collected and returned to the respective MBRs. The different tests were operated at different operational conditions that mentioned in Table 4.1. No sludge was withdrawn from the reactors during the experimental periods. The pre-cultures of *A. denitrificans* PR1 used as inoculum were grown in liquid MMBN medium (Reis et al., 2014). The PR1 cells were harvested by centrifugation (at 7000 xg for 10 min), washed 3 times with mineral medium before inoculating into the bioaugmented-MBRs.

Table 4.1. Conditions imposed in each experiment

Tests	HRT (h)	Operation days (d)	Acetate (mM)	SMX (µg L^{-1})	SMX shock (20 µg L^{-1})	Inoculation of PR1 in R1 reactors
M1	24	36	-	10	-	Day 1 (D1) (~10% v/v or ~31.3 $mg_{biomass}$ L^{-1})
M2	12	36	-	10	-	D1, D8, D22, D25 (~20% v/v or 62-67 $mg_{biomass}$ L^{-1})
M3	6	19	4	10	-	D1 (40% v/v or 150 $mg_{biomass}$ L^{-1})
M4	6	26	4	1	D3, D12, D19, D26	D1, D5, D12, D16, D19 (40% v/v or 105 to 129 $mg_{biomass}$ L^{-1})
M5	4	15	-	1	D7, D13	D1, D5 (40% v/v or 93 to 137 $mg_{biomass}$ L^{-1})

4.3.3. Chemical analysis

General reactor performance was monitored by analyzing influent (feed) and effluent (permeate) samples for soluble chemical oxygen demand (sCOD), total COD, ammonium, nitrite, and nitrate. Samples were centrifuged for 5 min at 8000 xg, followed by syringe filtration through 0.2 µm cellulose Whatman filters and then stored at -20ºC prior to analysis of soluble chemicals. Chemical oxygen demand (COD) was determined using HACH-lange test kits and a DR2800 spectrophotometer (HACH, Germany). Ammonium, nitrate and nitrite concentrations were measured using a segmented flow analyzer through the Skalar San++ Automated Wet Chemistry Analyzer system. Total and volatile suspended solids (TSS and VSS) were determined according to Standard Methods (APHA, 1995).

The acetate concentration was determined by high-performance liquid chromatography (HPLC) using an IR detector and a BioRad Aminex HPX-87H column. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.6 mL/min and a 50ºC operating temperature.

Analysis of SMX and its metabolite (Ac-SMX) was performed on a high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. Both standard addition (de Jesus Gaffney et al. 2015) and external calibration curve methods were used for the analyses of 10 µg L^{-1} and 1 µg L^{-1}, respectively. A detailed description of analytical methods used is provided as Appendix C1.

All analyses related to general reactor performance and SMX determination were performed at least three times a week. Analyses of SMX and Ac-SMX analyses were performed within one to two weeks to prevent degradation.

4.3.4. Molecular analysis

DNA extraction

Samples from *A. denitrificans* PR1 strain pure culture and MBRs mixed liquor samples of 5 mL were collected. Samples were centrifuged for 10 minutes at 9600 xg and supernatant discharged. Pellets were stored at -20ºC until further DNA extraction. MBR sampling was performed immediately after inoculation with PR1 strain and mixture in the reactor, and periodically until the end of the tests.

DNA was extracted with the Power Soil DNA Isolation kit (MoBio, Cambridge, UK). Negative controls for the extraction were included using sterile MilliQ water. DNA quality and concentration was analysed in duplicate with a Nanodrop 1000™ spectrophotometer (ThermoScientific, DE, USA).

Selection of the strain-specific genetic marker for A. denitrificans PR1

For the selection of the strain-specific genetic marker a comparative genomic analysis was performed between strain PR1 (Reis et al. 2017) and 7 other representative genomes of the genus *Achromobacter* (Table 4.2). All genomes were retrieved from the NCBI database (Database resources of the National Center for Biotechnology Information 2018) and re-annotated with Prokka (Seemann 2014). Predicted genes were analyzed with Roary with the minimum BLASTP identity percentage set to 80% (Page et al. 2015). This percentage of identity was chosen since these strains belong to different species and it was shown to be a suitable value in other comparative studies (Corretto et al. 2017). Genes unique to strain PR1 were further retrieved and aligned against the non-redundant nucleotide database of NCBI with BLASTN (Zhang et al., 2000). Genes producing no significant alignments to other published *Achromobacter* spp. genomes and with a low percentage of nucleotide identity (<80%) against the non-redundant database were further evaluated and the CRISPR-associated endonuclease (Cas1)

was selected as the strain-specific marker for this study (GenBank accession number CP020917.1, locus tag B9P52_11565).

PCR analysis

To quantify the PR1 abundance and consequently monitor its survival upon bioaugmentation in the MBRs, quantitative PCR (qPCR) targeting the strain-specific marker gene (Cas1) was performed with total DNA. Primers for both PCR and qPCR assays were designed with Primer-BLAST (Ye et al. 2012) and their specificity was further assessed *in silico* against the non-redundant nucleotide database from NCBI (Database resources of the National Center for Biotechnology Information 2018).

Two sets of primers were used: a first pair to obtain by PCR an amplicon to perform the calibration curves in qPCR with a transformed *Escherichia coli* and a second pair to quantify by qPCR the target gene in both transformed *E. coli* (for calibration) and *A. denitrificans* PR1 in MBR DNA samples.

For qPCR calibration, 6 primer pairs were designed targeting PR1 strain specific markers (Table 4.3). Amplification and reaction conditions for conventional PCR were tested for all six to choose the best primer pair. The specificity of such primers were confirmed by amplification of the target gene in the DNA extracted from a pure culture of *A. denitrificans* PR1, Sanger sequencing of the amplicon and by performing a BLAST of the obtained sequence against a non-redundant nucleotide database (EMBL-EBI 2017).

qPCR analysis

Three primer pairs were evaluated for qPCR amplification of the Cas1 gene by analyzing the match and the base pairing of each primer with the gene sequence obtained from the *A. denitrificans* PR1 DNA using EMBOSS Matcher and ClustalO software (both bioinformatics tools available from the EMBL-EBI site). Annealing temperature was further optimized for the best primer pair and amplification by qPCR was performed with the iQ5 real time system (BioRad, US), with the following conditions: initial denaturation for 5 min at 94 ^{o}C, followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and extension at 72 ^{o}C for 2 min, with a final extension of 72 ^{o}C for 5 min. The qPCR reaction was performed in 20 µl, containing 2 ng/µl of DNA template, 1x super mix iQ Syber Green and 0.2 µM if each primer. Reaction was performed in duplicates and the results reported as average of the measures with standard deviation. Data was analyzed in the form of standard curves and its parameters (efficiency, slope and R^2), melting curves and melting peaks and base line subtracting curves for each amplification experiment with the BioRad iQ5 optical system software (BioRad, US). The amount of target genes in unknown samples was calculated based on the standard curve retrieved from the series dilutions of transformed *E.coli* cells with the target genes (10^1 to 10^9 copy numbers/µl). Cas1 gene copy number were further normalized by the total DNA concentration of

each sample. Transformed *E.coli* was obtained by cloning of JM109 High efficiency competent cells, using the pGEM-T easy vector system, following the manufacturer's instructions (Promega, US). In the cloning process, PCR products to be ligated to the plasmid were purified with the MiniElute PCR Purification kit (Quiagen, Germany), the transformation success was double check by amplification with the qPCR Cas1 genes from the DNA extracted from the white colonies with the Quick DNA Universal kit (Zymo Research, US) and the plasmids were extracted using the ZR Plasmid miniprep (Zymo Research, US).

Table 4.2. Organisms and corresponding Genbank assembly accession numbers of the *Achromobacter* spp. strains used in the comparative genomic analysis

Species	**Strain**	**GenBank assembly accession number**
A. denitrificans	PR1	GCA_002205315.1
A. denitrificans	NBRC 15125^{T}	GCA_001571365.1
A. denitrificans	USDA-ARS-USMARC-56712	GCA_001514355.1
A. spanius	CGMCC9173	GCA_001189595.1
A. insuavis	AXX-A	GCA_000219745.1
A. xylosoxidans	A8	GCA_000165835.1
A. piechaudii	ATCC 43553	GCA_000164035.1
A. arsenitoxydans	SY8	GCA_000236785.2

Table 4.3. Primers targeting the selected strain-specific marker gene of PR1, CRISPR-associated endonuclease (Cas1)

PCR primers 5' - 3'	Product length (bp)	qPCR primers	5' - 3'	Product length (bp)
CRSP_f: GCCGACAGAAAAGCTTGC CRSP_r: CATTTATTACCTGGAGCATTGCC	789	Pair 1	Forward: AGTAGTTCCCATGATCGAGATAGCG Reverse: GGATATCACTGCCTTGCTTGCC	152
		Pair 2	Forward: TGGCCGACAGAAAAGCTTGCG Reverse: TCTCGATCATGGGAACTACTTGGC	176
		Pair 3	Forward: CCGCCTGTAGTTGCTTCGC Reverse: CCGAGTATCTACAGGCATGGG	78

4.3. Results and discussions

4.3.1. Reactor performance

The general MBR operating conditions and performance are presented in Table 4.4. During reactor operations, the biomass concentration decreased in the MBRs operated at an HRT of 24 h, whereas quite stable biomass concentration was observed in MBRs run at an HRT of 12 h and significant increase in biomass was found in the reactors at an HRT of 6 and 4 h, especially, with the amendment of acetate (Table 4.4).

Good COD removal was observed and there was no difference in COD removal efficiency between bioaugmented (R1) and non-bioaugmented (R2) MBRs operated under the same conditions (Table 4.4). Accordingly, the COD removal of the MBRs operated at HRT of 6 h (M3 and M4) and 12 h (M2) stabilized to between 85 and ~97% in both bioaugmented and non-bioaugmented MBRs, even with the addition of acetate (4 mM). No acetate was detected in the effluents of the reactors supplemented with acetate (M3 and M4).

Ammonium loading rate (ALR) was highest in M5 reactors whereas it was similar in M4, M3 and M2 reactors and the lowest one was in M1 reactors (Table 4.4). Complete removal of ammonia was observed in all reactors (an average effluent NH_4^+-N of < 2 mg L^{-1}), concomitant with the formation of nitrate $NO_3^- - N$ (no $NO_2^- - N$ was found), which indicates that stable and high nitrification performance occurred (Table 4.4). As can be seen from (Table 4.4)., the nitrification capacity per unit of biomass was highest for the reactors operated at HRT = 4 h (M5), supposedly due to the high ALR (lower HRT).

Nitrification rates in the reactors with HRT = 12 h (M2) were higher than the ones operated at HRT = 6 h (M3 and M4), indicating a higher activity of autotrophs (ammonia oxidizing bacteria). The reason could be attributed to the amendment of acetate to M3 and M4 which favored the growth of heterotrophs in these reactors, and thereby lowered the nitrification activity due to the higher quantity of ammonia assimilated for biomass growth.

Table 4.4. General reactor operating conditions and performances during the MBR tests (average ± standard deviation) (average = mean of samples taken over times)

	Acetate (mM)	HRT (hour)	Biomass (gTSS L^{-1})		sCOD (mg L^{-1})		OLR (g sCOD $gTSS^{-1}$ d^{-1})	$NH_4^+ - N$ (mg L^{-1})		ALR (mg N $gTSS^{-1}$ d^{-1})	$NO_3^- - N$ (mg L^{-1})	
			Initial	End	Influent	Effluent		Influent	Effluent		Influent	Effluent
M1-R1	-	24	2.82	2.20	96 ± 32	34 ± 5	0.04 ± 0.01	35.7 ± 12.0	0.8 ± 1.1	15.4 ± 5.5	0	21.8 ± 7.7
M1-R2	-	24	2.87	2.04	96 ± 32	35 ± 5	0.04 ± 0.01	35.7 ± 12.0	1.0 ± 2.3	16.3 ± 6.0	0	22.6 ± 5.5
M2-R1	-	12	3.36	3.30	105 ± 21	13 ± 5	0.07 ± 0.01	41.6 ± 5.7	0.4 ± 0.6	29.5 ± 4.7	0	48.0 ± 4.9
M2-R2	-	12	3.30	2.88	105 ± 21	14 ± 5	0.08 ± 0.02	41.6 ± 5.7	2.1 ± 5.7	30.3 ± 5.8	0	44.2 ± 9.9
M3-R1	4	6	3.52	8.04	377 ± 10	16 ± 2	0.30 ± 0.08	44.3 ± 4.9	1.1 ± 0.6	31.6 ± 9.4	0	36.0 ± 4.9
M3-R2	4	6	3.4	8.3	377 ± 10	18 ± 6	0.29 ± 0.09	44.3 ± 4.9	0.9 ± 0.5	32.6 ± 11.3	0	37.0 ± 4.4
M4-R1	4	6	3.43 ± 0.0	9.30 ± 0.1	376 ± 24	26 ± 5	0.29 ± 0.09	36.8 ± 5.3	0.7 ± 1.4	28.0 ± 4.95	0	32.6 ± 4.7
M4-R2	4	6	3.34 ± 0.1	10.20 ± 2.2	376 ± 24	24 ± 4	0.33 ± 0.07	36.8 ± 5.3	1.0 ± 1.9	32.5 ± 3.5	0	30.3 ± 5.7
M5-R1	-	4	2.55 ± 0.0	4.10 ± 0.0	103 ± 17	30 ± 12	0.17 ± 0.03	44.9 ± 3.4	7.5 ± 10.2	81.0 ± 22.0	0	33.5 ± 11.3
M5-R2	-	4	2.70 ± 0.1	4.50 ± 0.42	103 ± 17	22 ± 12	0.21 ± 0.09	44.9 ± 3.4	12.4 ± 13.5	97.5 ± 32.3	0	32.5 ± 13.3

OLR: organic loading rate; ALR: ammonium loading rate; R1-bioaugmented MBR; R2-non-augmented MBR

4.3.2. SMX removal efficiency

SMX adsorption tests using the same activated sludge was performed in Nguyen et al. (2018). In terms of mass balance, no SMX was removed during adsorption tests, suggesting that the adsorbed amount of SMX on activated sludge was negligible. This is also consistent with other studies where partitioning of SMX onto secondary sludge accounted for approx. 4% (Göbel et al., 2005) to 10% (Hörsing et al., 2011) of the total elimination efficiency. For that reason, adsorption of SMX was considered irrelevant and not a subject of investigation in this study.

Along with SMX, the concentration of human conjugated metabolite (Ac-SMX) in the feed and effluent of the MBRs was also monitored, ranging from 500 ng L^{-1} to ~ 1200 ng L^{-1}, and < 120 ng L^{-1}, respectively (data not shown). The decrease in Ac-SMX concentration was possibly due to the back-transformation to SMX (Göbel et al., 2005).

The SMX specific degradation rates were determined through SMX removal normalized by the biomass concentration (Figure 4.1B). As can be seen from Figure 4.1A, higher specific degradation rates and higher average SMX removal efficiencies were observed for the bioaugmented reactors compared to non-bioaugmented ones under all operational conditions. The average SMX removal varied under different operational conditions, ranging from 39% to 70% for non-bioaugmented MBRs (R2), and 48% to 87% for bioaugmented reactors (R1). A SMX removal efficiency of 52-64% was reported at HRTs of 12-14 h Taheran et al. (2016), which is consistent with our result obtained with a non-bioaugmented MBR operated under HRT of 12 h (56.5 ± 26% in M2-R2). Radjenović et al. (2009) reported a SMX removal efficiency of 78% in a hollow-fiber MBR with HRT of 7.2 h, which is comparable to the average removal of 67.5 ± 10.5% observed for the MBR operated under HRT of 6 h in this study (M3-R2).

Low SMX removal efficiencies concomitant with decreased MLSS concentrations were observed for the reactors operated at HRT of 24 hours (M1). The removal was only 48 ± 28% and 39 ± 20% on average, for bioaugmented and non-bioaugmented reactors, respectively. The reasons for such low removal efficiencies could be attributed to the low concentration of substrate in the reactor, or due to a decrease in biomass that could be limiting for SMX removal (discussed in section 4.3.3). Bioaugmentation did not result in improved SMX removal under this limiting condition. It was mentioned by Herrero and Stuckey (2015) that bioaugmentation was not successful under growth-limiting conditions due to low substrate concentration.

For the tests performed at 10 µg L^{-1} (M1, M2 and M3), a decreased HRT from 24 h to 12 h and then 6 h also resulted in increased specific degradation rates. The highest specific degradation rate was observed for the reactors operated at HRT of 6 h (M3), with the addition of acetate, which was about

6.4 ± 3.4 µgSMX gSS^{-1} d^{-1} and 4.9 ± 1.7 µgSMX gSS^{-1} d^{-1} for the bioaugmented and non-bioaugmented reactors, respectively.

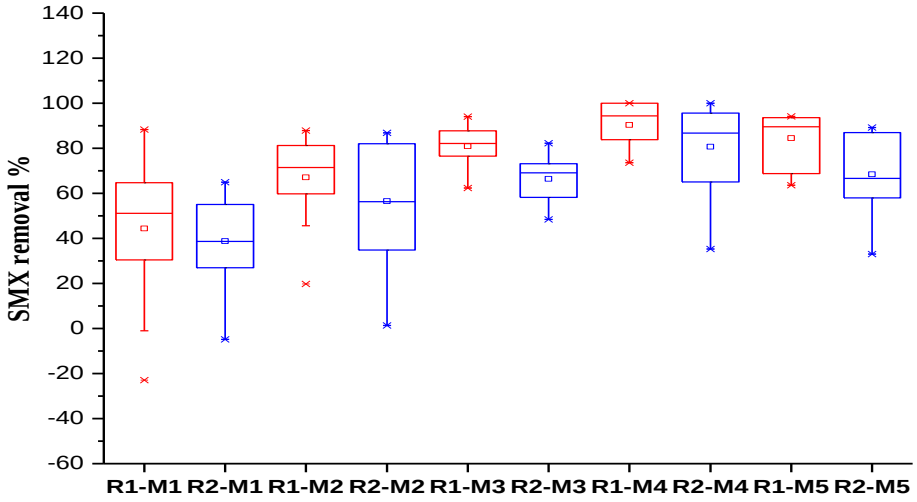
For the tests performed at 1 µg L^{-1} (M4 and M5), the presence of acetate led to a significant increase in biomass concentration in M4, and thus also a slight increase in SMX removal efficiency, but lower in SMX specific degradation rate compared to M5 (Figure 4.1). This indicates that the presence of acetate led to a significant increase in biomass concentration, although likely these were mostly non-specialized heterotrophs. These results suggest that, despite the fact that the degradation kinetics of SMX by PR1 was stimulated in the presence of acetate in pure culture tests (Nguyen et al., 2017), there is no advantage in practice to supplement bioaugmented AS with acetate as specific substrate to enhance the kinetic of SMX by PR1.

In terms of SMX concentration effect, a decrease in SMX concentration in the feed led to an increase in SMX removal efficiency. Higher SMX removal efficiency was observed for the M4 tests with 1 µg L^{-1} of SMX compared to the M3 tests performed with 10 µg L^{-1} of SMX (81 ± 18.1% vs 67.5 ± 10.5% and 90.4 ± 10 vs 80.9 ± 9.2 vs % for non-bioaugmented and bioaugmented reactors, respectively). (Al-Ahmad et al., 1999) found a 50% minimum inhibitory concentration (MIC_{50}) for SMX for common pathogens ranging from 0.002-256 mg L^{-1} SMX. Thus, the supplied concentration of 10 µg L^{-1} SMX in this study might influence microbial activity, resulting in lower SMX removal. Good SMX removal was observed even at such low SMX concentrations as 1 µg L^{-1}, suggesting that either any existent threshold levels for the removal of SMX was below this concentration or cometabolism seems to be the removal mechanism of SMX, in which primary substrates play an important role in the biological removal of micro-pollutants by inducing enzymes or supplying energy for biomass growth and maintenance. Such threshold levels are typical from biodegradation mechanisms where the target compound is a source of carbon and/or energy. Metabolic and cometabolic transformations were previously observed as the biodegradation mechanisms of SMX by PR1 (Nguyen et al., 2017) and AS (Kassotaki et al., 2016; Müller et al., 2013).

In general, the introduction of PR1 into the MBR systems enhanced SMX removal and degradation rate in comparison to the non-bioaugmented MBRs, which can be explained by the fact that the inoculated PR1 was specific for the target compound (SMX). Many isolates, such as *Acinetobacter sp.*, *Phanerochaete chrysosporium,* and *Microbacterium sp.* etc., are capable of SMX degradation with high removal efficiency (Wang and Wang, 2018). However, previous studies on SMX biodegradation by pure culture were usually performed at rather high concentration of SMX, in the range from several mg L^{-1} to hundreds mg L^{-1} that is much higher than the concentrations encountered at WWTPs. Some bacteria failed to degrade the contaminants at environmentally relevant concentrations even if they have that capability (de Lipthay et al., 2007; Xu et al., 2009), probably, due to the lack of induction of the corresponding catabolic genes (Kolvenbach et al., 2014). For example, no SMX degrada-

tion was reported for bioaugmentation of MBR with a *Microbacterium* sp. strain BR1 for treatment of SMX in two pilot-scale MBR treating full-scale MBR effluent (post-treatment) (Fenu et al., 2015). The reason was attributed to the very low SMX concentrations, which could be limiting for the inoculated biomass.

1A



1B

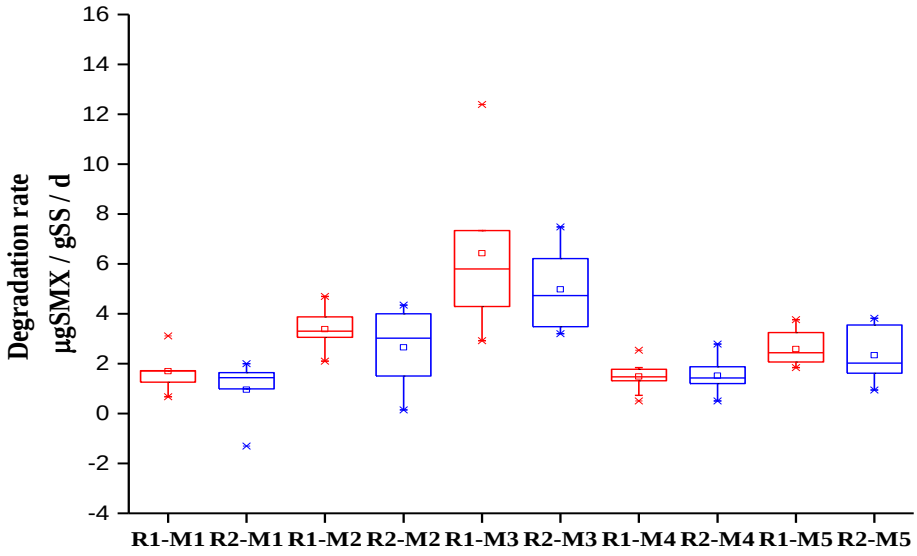


Figure 4.1. SMX removal efficiences (1A) and SMX specific degradation rates (1B) (*not counted for the shock loading samples*) in the bioaugmented (R1) and non-bioaugmented (R2) reactors operated under different conditions, e.g. HRT = 24 h (M1); HRT = 12 h (M2); HR

The relatively high SMX removal observed even in the non-bioaugmented reactors (except for the MBR operated under a HRT of 24 h) could be explained by the superior performance of MBR compared to conventional AS. The antibiotics removal efficiency in MBR processes was reported to

be 15-42% greater than in conventional AS processes (Sahar et al., 2011), due to the long SRT that favors the proliferation of slowly growing bacteria (such as nitrifying bacteria), thus improving the microbial diversity in the reactor and achieving better biodegradation (Clara et al., 2005b; Radjenović et al., 2009).

Since the occurrence of SMX in WWTP vary over a wide range from hundreds ng L^{-1} (Watkinson et al., 2007) to µg L^{-1} (Batt et al., 2007) and even as high as 7910 µg L^{-1} (Peng et al., 2006), we hypothesized that SMX concentration fluctuations could also affect the SMX removal efficiency in biological treatment. In the current study, shock loads of SMX (20 µg L^{-1}) were applied in some days during the M4 and M5 tests to evaluate their effect on removal efficiency. As can be seen in Figures 4.3A and 4.3B, SMX shocks inhibited SMX removal in non-bioaugmented reactors (R2), resulting in decreased or negative SMX removal efficiencies. The negative efficiency in the non-bioaugmented MBR could be attributed to the accumulation of SMX from the previous day when a SMX shock load was applied (Figures 4.3A and 4.3B). In contrast, bioaugmented reactors (R1) were more resistant to SMX shocks (Figures 4.3A and 4.3B). It has been demonstrated that bioaugmentation could protect the reactors from sudden toxic pollutant shock loads and allow recovery of functionality for other pollutants (Boon et al., 2003; Qu et al., 2009).

4.3.3. Effect of HRT and loading rates on SMX removal

The results of the current study showed that higher average SMX removal were observed for the tests performed at HRT of 6 hours (M3) compared to 12 hours (M2) and 24 hours (M1) (Figures 4.2A, 4.2B and 4.2C for both R1 and R2 reactors. Two sample t-tests revealed that the average SMX removal in the reactors operated at HRT=24 h was significantly lower than in the HRT=12 h treating similar influent wastewater ($p = 0.007$ for the non-bioaugmented reactor and $p=0.002$ for the bioaugmented reactor). The good SMX removal observed when the HRT reduced from 24 h to as low as 4 h, indicated that a relatively low contact time was sufficient to treat the SMX in wastewater. Nevertheless, HRT has an influence on SMX removal, rather due to the availability of primary substrates (organic compounds and ammonia loading) in the reactor. The fact that both bioaugmented and non-bioaugmented tests performed worse in terms of SMX removal efficiencies at HRT of 24 h suggests that the presence of primary substrates can favor the cometabolism of SMX by the inoculated strain as well as AS bacteria. In this study, a correlation between the SMX removal capacity and the OLR was observed in the M3, M4 and M5 tests, along with a correlation with the ammonium loading rate (Figures 4..4, 4.5 and 4.6). This finding agrees well with previous studies (Fernandez-Fontaina et al., 2012; Ren et al., 2007; Su et al., 2015). These authors also found links between organic matter removal or nitrification capacity and micropollutants removal efficiency. These results also suggest that the removal rates of SMX in AS processes are strongly linked to both the autotrophic and heterotrophic bacterial communities. Given that SMX biotransformation has been demonstrated to involve both metabolic and co-metabolic

mechanisms (Kassotaki et al., 2016; Müller et al., 2013), degradation of SMX at such low levels require the presence of primary substrates, e.g. easily biodegradable substrates or ammonia, to induce catabolic enzymes (specific and non-specific ones) or to supply energy for cell growth and maintenance (Nguyen et al. 2017). The higher the consumption of primary organic substrates (as a result of high OLR) or ALR, the higher the rate of SMX cometabolised is expected.

For the tests with 1 μg L^{-1} of SMX, with the amendment of acetate in the M4 reactor, lowering HRT from 6 h (M4) to 4 h (M5) resulted in lower OLR but higher ALR in reactors M5 compared to M4. Consequently, the observed higher SMX removal rates in M5 compared to M4 was supposedly not caused by differences in the heterotrophic bacterial community, but rather to a higher nitrification capacity or a higher density of ammonia oxidizing bacteria. Thus, the amendment of acetate seemed unnecessary for the removal of SMX by both AS and introduced strain PR1.

Long HRT implies longer contact time between wastewater or contaminants and sludge, which may be beneficial for the general slow removal rate micropollutants, but not for SMX in this study. Short HRT favours higher rate wastewater treatment processes and implies that more wastewater can be treated per reactor volume unit as compared to long HRT systems, which is advantageous for high-rate wastewater treatment processes. Maurer et al. (2007) also found a quantitative correlation between elimination rate of the investigated β-blockers and HRT. However, Taheran et al. (2016) compared the removal efficiencies reported by different researchers and observed that HRT had no effect on removal of acetaminophen, bezafibrate, ofloxacin, gemfibrozil and metronidazole. The difference in conclusions could probably be attributed to the difference in the chemical structures or microbial removal mechanisms.

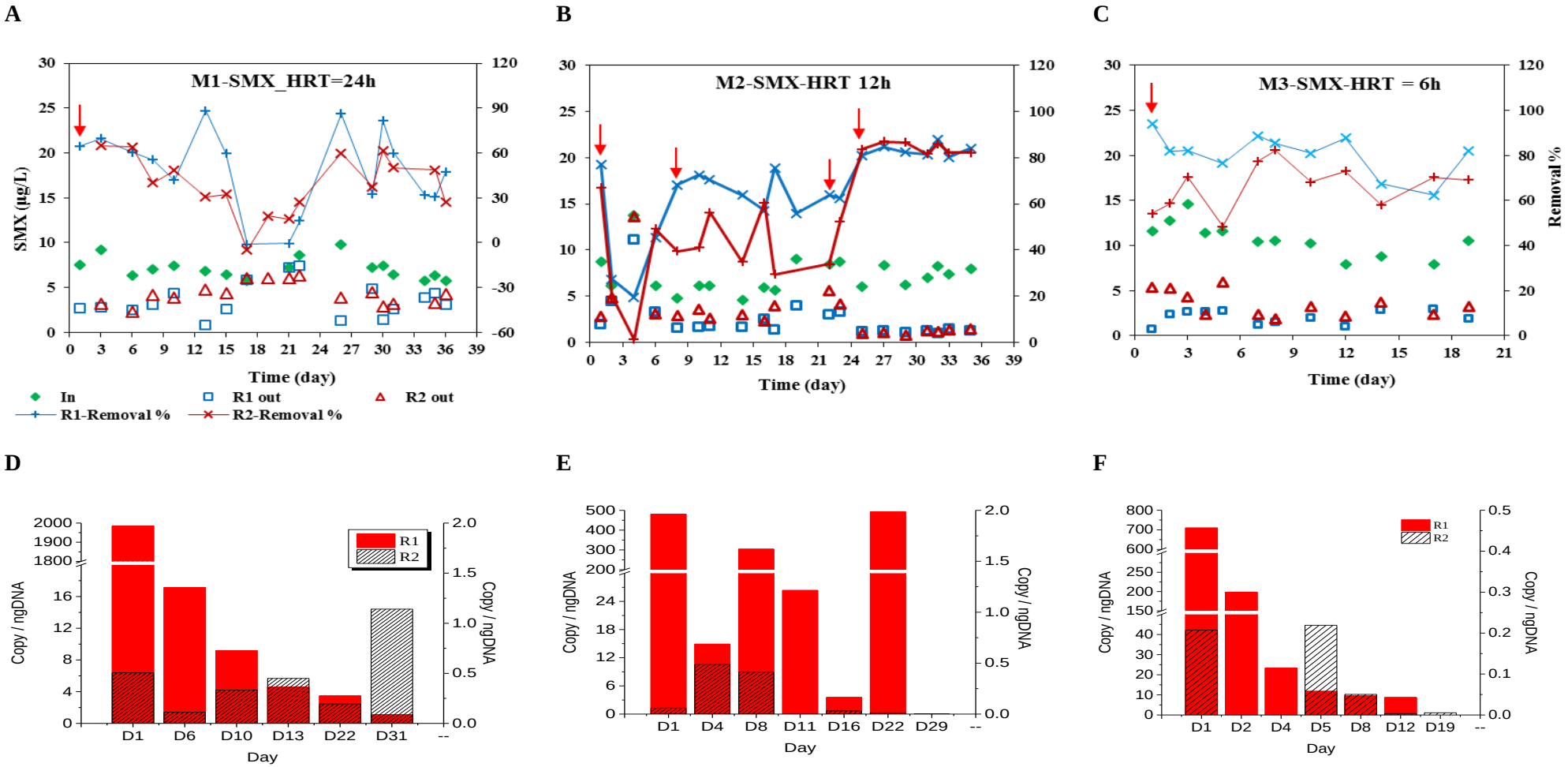


Figure 4.2. Degradation of SMX and fate of PR1 assessed by qPCR for the 10 µg L^{-1} of SMX tests, under different operational conditions, i.e. M1 (HRT=24h, single inoculation of PR1), M2 (HRT=12h, multi-inoculation of PR1), M3 (HRT=6h, with addition of acetate, single inoculation of PR1) in bioaugmented MBR (R1) and non-bioaugmented MBR (R2)

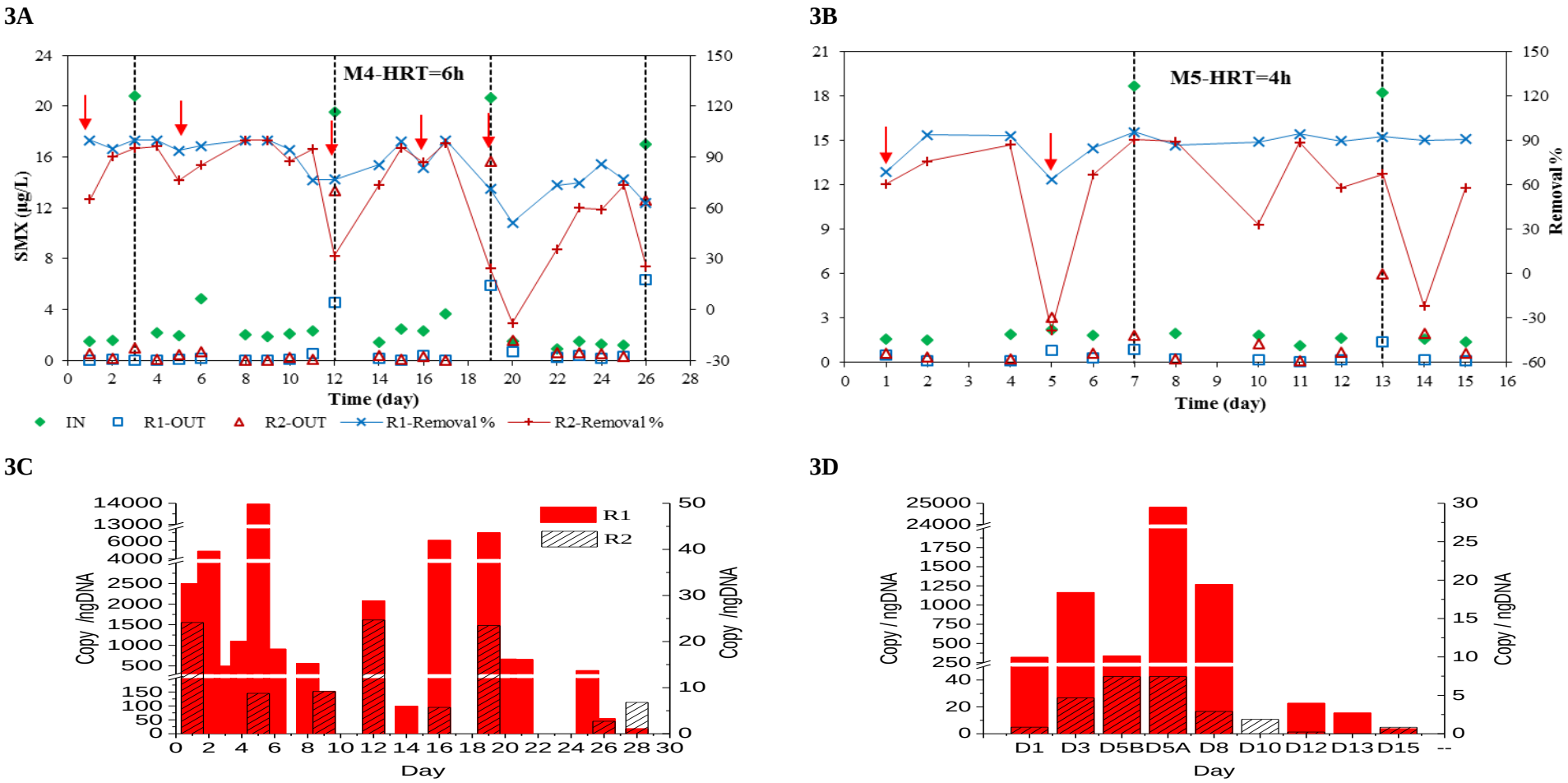


Figure 4.3. Degradation of SMX (3A and 3B) and fate of *A. denitrificans* strain PR1 (3C and 3D) assessed by qPCR for the 1 µg L^{-1} of SMX tests under different operational conditions, e.g. M4 (HRT=6h, with addition of acetate, multi-inoculation of PR1), and M5 (HRT=4h, multi-inoculation of PR1) in bioaugmented-MBR (R1) and non-bioaugmented MBR (R2). The dashed lines mark the times when SMX shock loads were applied. Inoculation points were indicated by the red vertical arrows.

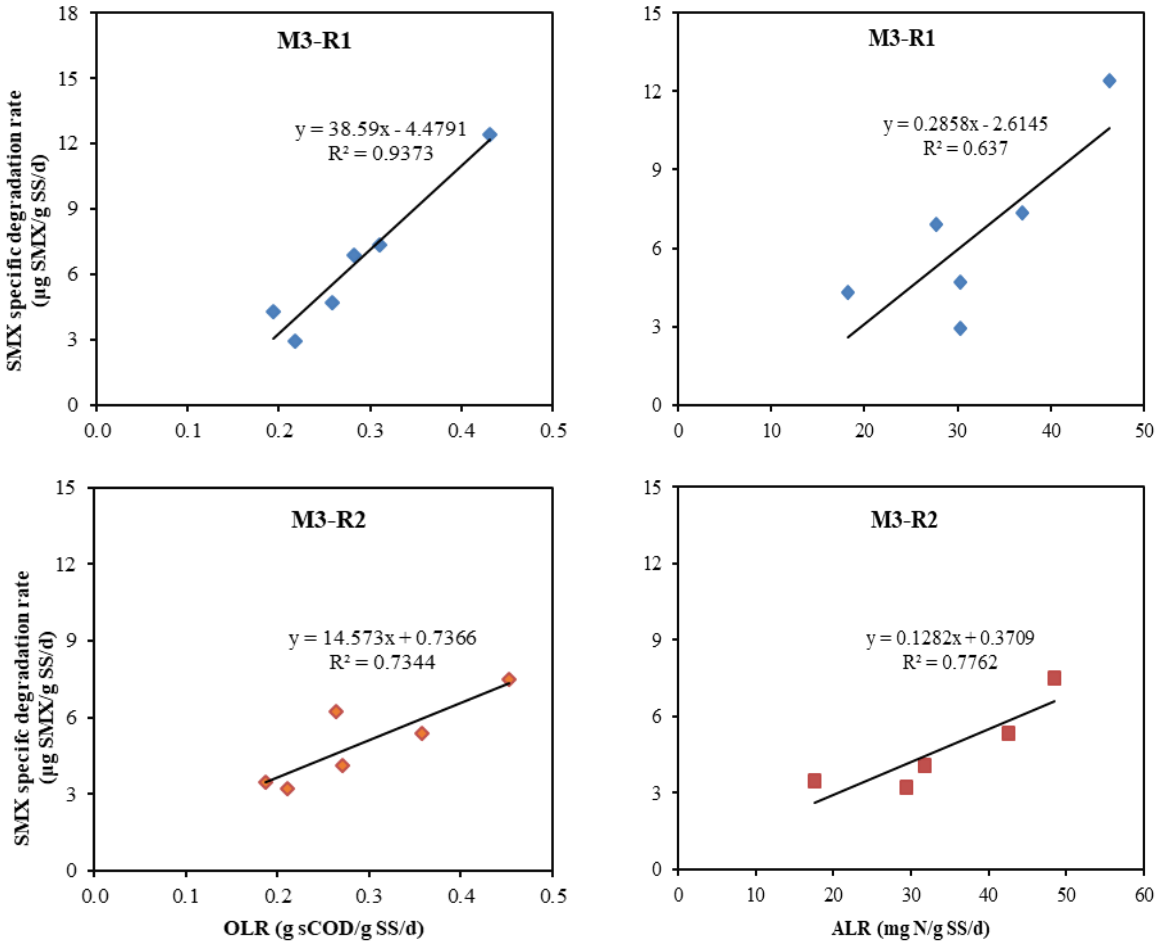


Figure 4.4. Relationship between specific SMX degradation rate, OLR and ALR for bioaugmented (R1) and non-bioaugmented (R2) reactors under HRT = 6 h, with acetate, single inoculation of PR1 (M3).

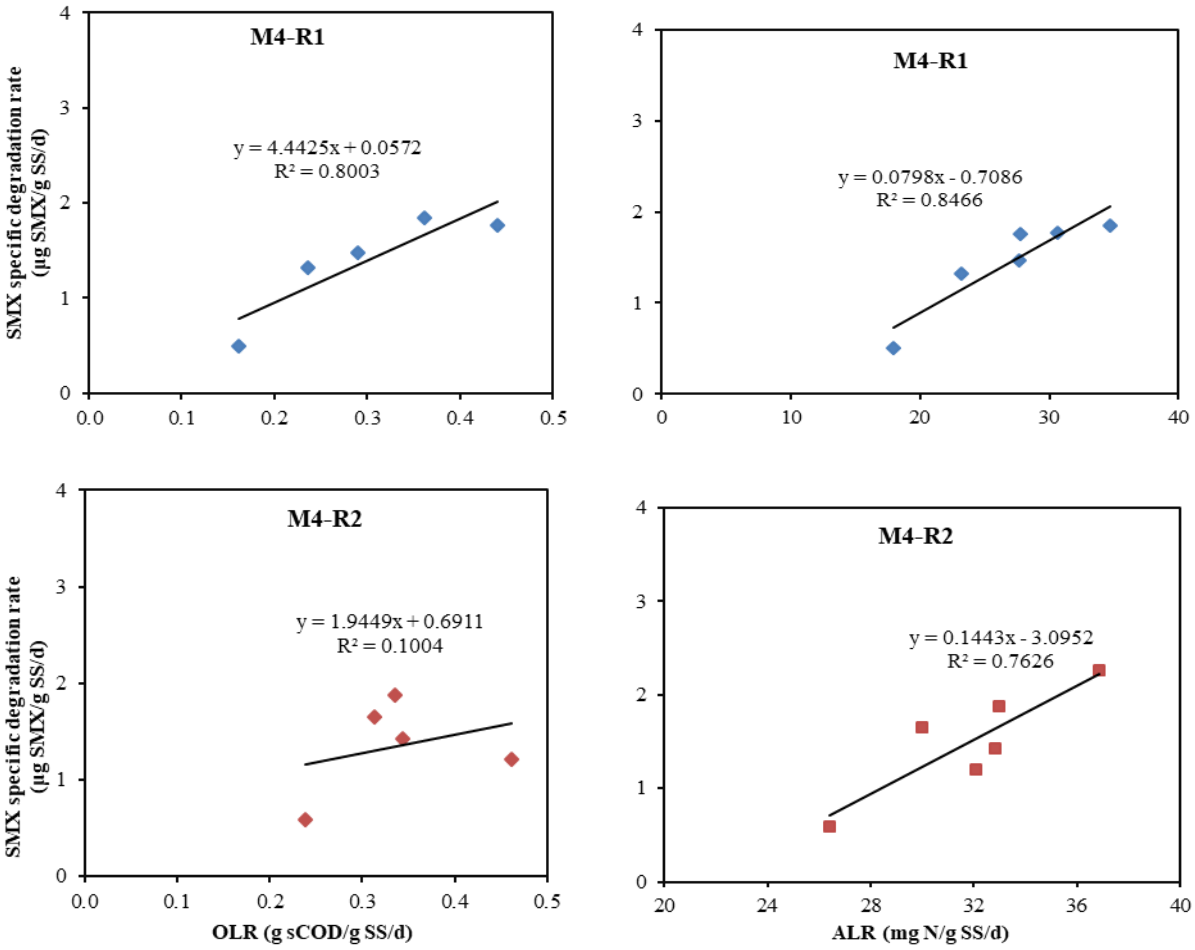


Figure 4.5. Relationship between specific SMX degradation rate, OLR and ALR for bioaugmented (R1) and non-bioaugmented (R2) reactors under HRT = 6h, with acetate, multi-inoculation of PR1 (M4)

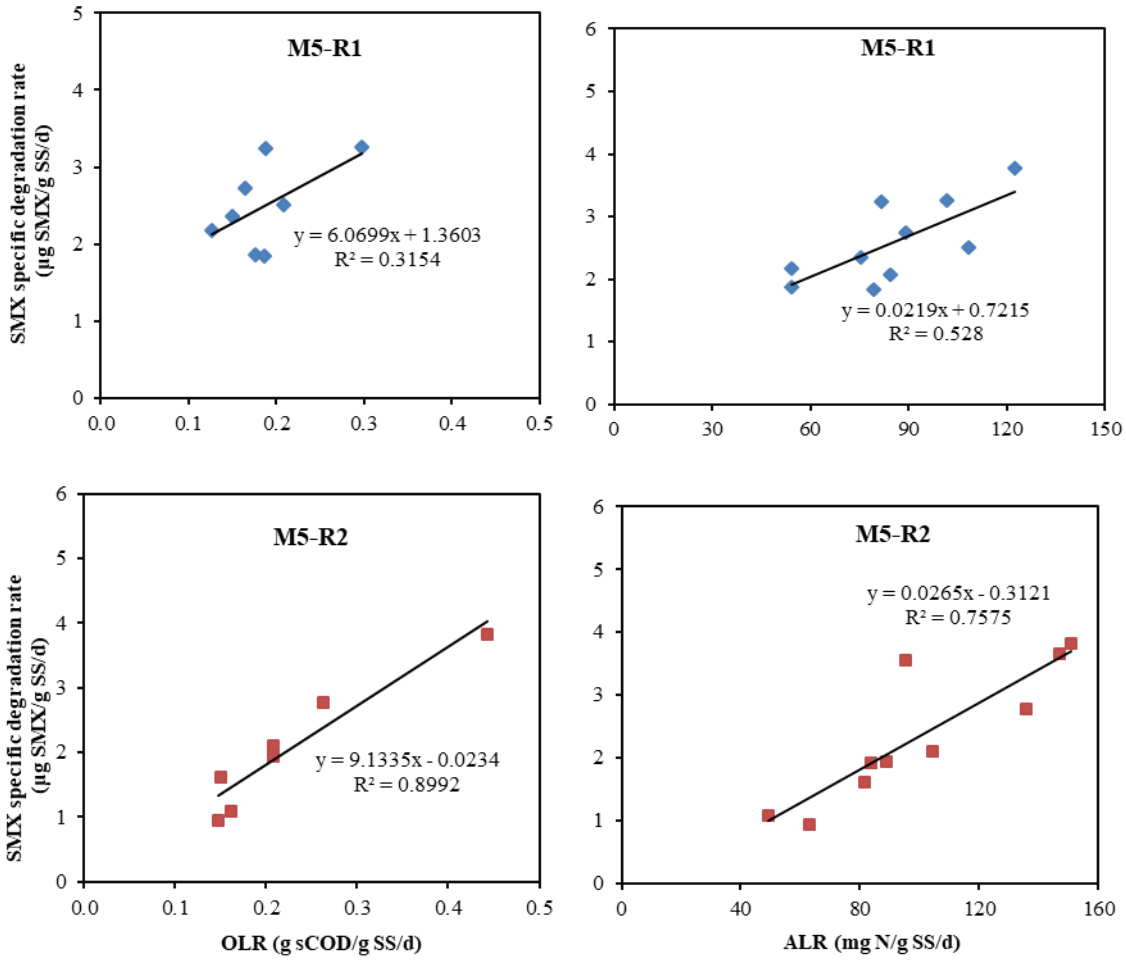


Figure 4.6. Relationship between specific SMX degradation rate, OLR and ALR for bioaugmented (R1) and non-bioaugmented (R2) reactors under HRT = 4h, multi-inoculation of PR1 (M5)

4.3.4. Survival and activity of A. denitrificans PR1 in the MBRs

During the bioaugmentation experiments, the survival of the introduced bacteria, *A. denitrificans* PR1 suspended cells, in the bioaugmented reactors was followed up by analyzing the evolution of the copy number of its marker gene (Cas1) which was monitored by qPCR.

Bioaugmentation for micropollutants removal is considered successful when multiple criteria are met, e.g. the bioaugmented strains can grow and remove the micropollutants to levels below typical WWTP effluent levels within a complex substrate background, such as wastewater, at practical degradation rates (Zhou et al., 2013). Overall in these experiments, a decrease in Cas1 gene copy number was observed, indicating a decrease in PR1 cell numbers over time. Non-bioaugmented reactors showed negligible levels of Cas1 gene, fluctuating between 0 to 24.7 copies/ngDNA, suggesting that the target gene was a good indicator to monitor the fate of the augmented strain in this study. Single inoculation was applied for M1 (HRT=24 h) and M3 (HRT=6 h, with addition of acetate) experi-

ments. For the bioaugmented reactor M3, a drastic drop in the associated gene was observed, from approx. 710 copies/ngDNA on day 1 to only about 200 copies/ngDNA on day 2 (Figure 4.2F), followed by a gradual decrease during the rest of the experiment to not detectable levels at the end of the experiment (after 19 days). A similar trend was observed in the bioaugmented reactor M1, e.g. only 1 copy/ngDNA was quantified on day 31 (Figure 4.2D).

Since the use of a membrane allows minimizing the washout of microbes, the loss of viability of the introduced bacteria was likely governed by other factors. It could be attributed to the diverse environmental stresses, i.e. predation by protozoa, intense competition with other bacteria, and unavailability of nutrients and oxygen (Gentry et al., 2004). Other studies also demonstrated that bioaugmentation, even using a strain originated from a similar ecosystem and able to effectively grow on the selective substrate, such as the one used in this study, is not permanent and will probably require regular re-supplementation (Boon et al., 2000).

The poor level of survival of the inoculated strain observed in experiments M1 and M3 suggested that a regular re-inoculation of the specialized strains could increase the success of bioaugmentation. Thus, repeated inoculations of freshly grown metabolically active cells were applied for the bioaugmented (R1) reactors M2, M4 and M5, in order to maintain the viability and metabolic inoculum activity of the strain. With re-inoculations, higher and more stable SMX removal levels were attained in M2, M4 and M5 reactors compared to M1, especially when subjected to SMX shock loads. However, a lower SMX removal rate was observed for re-inoculated reactor M2 compared to single inoculated reactor M3. These results suggested that, rather than re-inoculation, the increase in removal efficiencies were more likely due to the change in HRT which led to a higher concentration of substrates available and favored the activity of the inoculated strain. The repeated inoculations with high inoculum concentrations in M2, M4 and M5 reactors also were followed by drastic decreases in Cas1 gene copy numbers shortly after inoculations and resulted in very low numbers at the end of the experiments (Figures 4.2E, 3C and 3D). Previous studies showed that repeated inoculations with massive cell numbers would increase the population of predatory protozoa, resulting in a rapid decline of the inoculated strains and sensitive indigenous bacteria, even resulting in system breakdown (Bouchez et al., 2000). However, the repeated inoculations themselves in this study apparently did not cause any disruption in function of the AS biological performance, e.g. COD and nitrogen removal.

During the first inoculation in M5 (R1) reactor, a significant increase in Cas1 copy number was observed after 3 days inoculation, then stabilized to the initial concentration after 5 days before the addition of the second inoculation. M4 reactor also showed increases in Cas1 number on day 2 and day 4. This suggests that operation at HRT of 4 h could be the most favorable condition for the survival of PR1 compared to the other HRTs. The reason could be due to the higher loads of primary sub-

strates that support the growth of the strain. Higher removal efficiency of SMX was observed at this HRT, which suggests that this could be the most favorable operational condition.

In all of the reactors, the amount of marker gene copies decreased significantly over time which resulted in very low to almost non-detected levels at the end, but higher SMX removal efficiencies and specific degradation rates were observed in all bioaugmented reactors (R1) compared to non-bioaugmented ones (R2), except for the M1 reactor. To explain this observation, we assumed that the growth of biomass could contribute to dilution and thus variation in quantification of the Cas1 marker. During reactor operation, a significant increase in biomass was found in reactors at HRT of 6 and 4 hours (M3, M4 and M5), especially with the amendment of acetate (Table 4.4). Moreover, the specific growth rate (μ) of *A. denitrificans* PR1 with acetate (2.2-2.3 d^{-1}) (Nguyen et al., 2017) was observed to be lower than that of AS heterotrophs (6 d^{-1}). Thus, the strain could be outcompeted by the heterotrophic population, resulting in a low relative copy number of Cas1.

From the current study as well as other studies, the introduced strain population was found to decrease shortly after being inoculated due to several abiotic and biotic stresses. To improve the bioaugmentation efficiency, immobilization of specialized consortia has been reported as a strategy for maintaining efficient degradation in wastewater treatment (Bouchez et al., 2009; Qu et al., 2006). However, immobilization of degrading strains within alginate beads or polyvinyl alcohol etc. may be costly and complex for practical application. To overcome this problem, immobilization of specialized degraders into biofilms reactors has been found to enhance the degradation of toxic pollutants in wastewater (Fang et al., 2013; Wang et al., 2012). It is proposed that further studies on immobilization of the specialized degraders onto biofilm carriers in a MBR (biofilm membrane bioreactor-BF-MBR) could improve survival of newly introduced strains in bioaugmented systems, thus improving the bioaugmentation efficiency.

4.4. Conclusions

In the current study, MBRs were bioaugmented with *Achromobacter denitrificans* strain PR1 to enhance the removal of SMX from wastewater and compared to a control, non-bioaugmented reactor. Influences of different operational condition, e.g. HRT and effect of addition of specific substrate (acetate) on the efficiency of bioaugmentation were investigated. Based on the results, the following conclusions could be drawn:

- HRT had an impact on SMX removal, and high HRT results in low F/M that could be limiting for SMX removal. Decreases in HRT from 24 h to 12 h, 6 h and finally 4 h resulted in an increase in SMX removal efficiency and specific degradation rate, which is likely due to increase in primary substrates availability, e.g. organic compounds and ammonia.

- Positive correlations between OLR or ALR and SMX removal were found, indicating that the presence of primary substrates are important for enhancing SMX degradation by AS and the inoculated strain.
- Bioaugmentation of PR1 resulted in enhanced and stabilized SMX removal, especially when SMX shock loads occurred. MBR could be useful in preventing washout of biomass but other factors, e.g. predation by protozoa, competition by indigenous bacteria etc. could govern the observed loss of the introduced strain over time. Despite the decrease in abundance of the introduced strain after a relatively short period of time, the bioaugmented reactors were generally more efficient in SMX removal than their non-bioaugmented counterparts. The fact that SMX is widely present in wastewater, rarely fully removed from WWTPs, and PR1 is very efficient in SMX removal (particularly at the low SMX levels normally found at WWTPs), show that bioaugmentation with PR1 is a viable operational strategy to enhance removal of SMX in poor performing WWTPs or in hospital/pharmaceutical wastewater treatment if more stringent discharge limits for SMX will be set to reduce the unwanted release of antibiotics into the environment.
- Re-inoculation of the degrading strain seemed not to be the suitable solution and immobilization onto biofilm carriers in a MBR is suggested for further studies to maintain survival of bioaugmented strains and removal efficiency of the target compound.

4.5. Acknowledgement

This research was supported by MERMAID, an Initial Training Network funded by the People Programme (Marie Skłodowska-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°607492, and by UCIBIO (FCT/MEC UID/Multi/04378/2013, POCI-01-0145-FEDER-007728). And by projects: (i) POCI-01-0145-FEDER-006939 (Laboratory for Process Engineering, Environment, Biotechnology and Energy – UID/EQU/00511/2013) funded by the European Regional Development Fund (ERDF), through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) and by national funds, through FCT; (ii) NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO-INNOVATION, supported by North Portugal Regional Operational Program (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the ERDF. Ana Reis wishes to acknowledge the Portuguese Foundation for Science and Technology (FCT) for her PhD scholarship (grant reference: SFRH/BD/95814/2013). iNOVA4Health Research Unit [grant number LISBOA-01-0145-FEDER-007344], cofunded by Fundacao para a Ciencia e Tecnologia/Ministerio da Ciencia e do Ensino Superior, through national funds, and by FEDER under the PT2020 Partnership Agreement, is acknowledged. Ana Filipa Silva wishes to acknowledge the Portuguese Foundation for Science and Technology (FCT) for her PostDoctoral scholarship (grant reference: SFRH/BPD/97612/2013).

5

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Thesis outcomes:

The main focus of this work was to investigate the potential for enhanced removal of the antibiotic SMX from WWTPs, using bioaugmentation with *Achromobacter denitrificans* strain PR1.

During the course of this PhD study, the major accomplishments of this work were:

- Characterization of the kinetics of SMX degradation by *A. denitrificans* strain PR1 in pure culture under environmentally relevant concentrations (Chapter 2).
- Characterization of the kinetics of SMX degradation by activated sludge as well as in bioaugmented activated sludge using *A. denitrificans* strain PR1 (Chapter 3).
- Understanding the fate of the two human metabolites, i.e. Ac-acetyl-SMX and sulfamethoxazole-N1-glucuronide, during biological processes (Chapter 3).
- Understanding effects of redox potentials, e.g. aerobic and anoxic, on the biotransformations of SMX and the two human metabolites by both AS and bioaugmentation degrading strain PR1 (Chapter 3).
- Pseudo-first order and cometabolic models were successfully calibrated to describe the removal of SMX and the two human metabolites in the systems (Chapter 3).
- Enhancing SMX removal in wastewater was achieved with bioaugmentated MBRs (Chapter 4)
- Understanding the effects of operational condition, e.g. HRT, as well as primary substrates (organic carbon or ammonium) on the SMX removal performances of bioaugmented and non-bioaugmented systems (Chapter 4)

- Contribution on development of qPCR method to monitor the traits of introduced strain PR1 in the bioaugmented MBRs (Chapter 4).

The main outcomes of this thesis are summarized as follows:

In this study, sulfamethoxazole degrading bacteria, *A. denitrificans* strain PR1, previously isolated from activated sludge and found to have capability to degrade and use SMX as sole source of carbon, nitrogen and energy, was assessed for its feasibility towards bioaugmentation applications.

Firstly, the SMX degradation kinetics of the strain were characterized. The strain was found to have the potential to degrade the antibiotic sulfamethoxazole (SMX) over a wide range of concentrations (ng L^{-1}, µg L^{-1} and mg L^{-1}), whereby SMX degradation kinetics were dependent on and stimulated in the presence of biogenic substrates, i.e. acetate or succinate. The biotransformation rate constant k_{bio} of the strain was 2 to 3 orders of magnitude higher than non-augmented activated sludge, which suggested that the strain is a potentially interesting organism for bioaugmentation to achieve SMX removal.

Experimental results from activated sludge batch tests suggested that retransformation of conjugate metabolites to the SMX in activated sludge occurred under both aerobic and anoxic conditions, which likely explains the previously observed negative or variability in SMX removal efficiencies in other studies. The results also demonstrated that biotransformation kinetics of SMX by activated sludge can vary significantly depending on redox conditions, i.e. SMX was biotransformed only under aerobic conditions. Notably, SMX transformation was enhanced when PR1 was bio-augmented in activated sludge. The addition of acetate as biogenic substrate is not necessary as the strain could use the carbon sources present in wastewater as biogenic substrates to achieve a sufficiently interesting SMX removal rates. The SMX biotransformation kinetics of PR1 was about sixty-times and hundred-eighty-times higher than the activated sludge in the presence and absence of primary substrates, respectively. These results prospect the use of *Achromobacter denitrificans* PR1 for bioaugmentation as a feasible and efficient strategy to improve SMX elimination in WWTPs. Biological degradation models such as the pseudo-first order kinetic and co-metabolism models, were successfully applied and the estimated kinetic parameters could describe well data measured of the biotransformations of SMX and the two human metabolites in bioaugmented and non-bioaugmented activated sludge batch experiments under various redox conditions.

Bioaugmentation of PR1 in membrane bioreactors for a long-term resulted in enhancing and stabilizing the removal of SMX, especially under SMX shock loads compared to the non-bioaugmented MBRs. However, the bioaugmentation strain could be sustained in the reactor only for a limited time, a maximum of 31 days. MBRs could be useful in preventing washout of biomass but other abiotic and biotic stresses, e.g. predation by protozoa, competition by indigenous bacteria etc.,

probably governed the decrease of the introduced microbial population. We found that, by changing operational conditions such as HRT, SMX removal could improve in wastewater in both bioaugmented and non-bioaugmented reactors. A correlation between the SMX removal efficiency and the OLR or ALR suggested that the removal rates of SMX in AS processes are strongly linked to the cometabolism of both autotrophic and heterotrophic bacterial communities, and that the higher the consumption of primary substrates or ammonium, the higher the observed rate of SMX cometabolized. High HRT (24h), resulting in low F/M, could be a limiting factor for SMX removal. Relatively good removal of SMX was found with the non-bioaugmented activated sludge investigated in this study, which could be attributed to (i) the superiority of advanced membrane technology compared to conventional activated sludge. Antibiotics removal in MBR processes was reported to be 15-42% greater than conventional activated sludge processes (Sahar et al. 2011); or (ii) favorable operational conditions applied in this study, e.g. no sludge withdrawal that favor the proliferation of slowly growing bacteria (such as nitrifying bacteria), thus improving the microbial diversity in the reactor and achieving better biodegradation. The fact that MBR is not applied at all WWTPs and the compound is ubiquitously present in the environment, with high detection frequency with incomplete and inconsistent SMX removal efficiencies in conventional WWTPs (Clara et al., 2005b, 2005a; Miège et al., 2009; Radjenović et al., 2009), suggests that bioaugmentation is needed to enhance removal of SMX in poor performing WWTPs or in hospital/pharmaceutical wastewater treatment.

The concentrations of SMX in our experiments are comparable with the concentration ranges that occurred in WWTPs and the reactors were also operated with real wastewater collected from a municipal wastewater treatment plant, and hence we can assume that our bioaugmented reactors were operated under realistic conditions. The proposed bioaugmentation MBR could be directly applicable to full-scale WWTPs to improve SMX removal performance. Also, knowledge of the effects of operational conditions such as HRT or loading rates (organic carbon and ammonium loading rates) will aid operators in determining ways to enhance their plant performance, through the manipulation of the HRT or OLR and ALR. Additionally, biokinetic characterizations of the bioaugmentation strain and activated sludge under different redox potentials are useful parameters and could be incorporated into the International Water Association (IWA) Activated Sludge Model to facilitate modelling the impacts of wastewater characteristics and operational conditions on SMX removals and allowing process optimization.

Recommendations for Future Research/Future perspectives:

During the course of this Ph.D study, a number of other issues were raised that call for further investigation. Recommendations for future research in this field are described below:

- *A. denitrificans* strain PR1 was previously isolated from activated sludge and found to be capable of removing many sulfonamide antibiotics, including SMX degradation with the stoichiometric accumulation of 3A5MI (Reis et al., 2014). Further studies are needed to elucidate the SMX degradation mechanism by PR1 as well as the enzymes/genes that are responsible for SMX degradation by this strain. The identification of the enzymes(s) responsible for SMX degradation could also provide a useful marker gene for monitoring gene expression upon bioaugmentation using *in situ* PCR or reverse transcriptase-PCR of extracted total RNA. These observation techniques can be used to monitor the activity of cells within a mixed microbial community and investigate which modification can have an influence on the metabolic activity of the introduced strain.
- It would also be interesting to understand the microbial communities of initial activated sludge and which populations are responsible for SMX removal. Stable isotope probing (SIP) has been considered as a potentially powerful tool to determine exactly which organisms assimilate specific contaminants (Radajewski et al., 2000). By understanding the specific degraders, biostimulation, which involves the identification and adjustment of factors such as nutrients that may be limiting the biodegradation rate of the contaminants by the indigenous microorganism at the affected site, could be applied for bioremediation of the targeted contaminants if applicable.
- Several MPs, e.g. diclofenac, hormones, and three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), were added to the Watch list of Decision 2015/495/EU and improvement in removal of these compounds is required if stringent discharge limits are imposed as anticipated. Advanced treatments, e.g. advanced oxidation processes, can remove these MPs very efficiently, but imply very high cost. Nowadays, since the tendency exists to apply "green" technology, bio-treatment is often a more economically feasible alternative. However, many of these MPs are still not fully removed in WWTPs, many of them due to their artificial nature, not easily degraded by natural enzymatic systems. Also, their biological degradation rates are very slow, thus requiring the enlargement of biological treatment tank volumes, which is not practically feasible. When operated successfully, bioaugmentation still holds the promising, cost-effective and sustainable biological abatement for fostering degradation rates of these MPs in wastewater. In this study, SMX was considered as a model pharmaceutical to which bioaugmentation was applied to enhance the removal efficiency in wastewater treatment. This research could be extended for enhanced removal of many other organic MPs in wastewater, e.g. diclofenac, natural and synthetic estrogens, etc. A mixed culture of "superbugs" with the ability to degrade targeted MPs simultaneously could be introduced to the activated sludge systems to enhance removal of the compounds under concern.

From an application perspective, bioaugmentation of the systems using a consortium was found to be more effective compared to the isolated one (Yao et al., 2013), as it provides the metabolic diversity and robustness needed for field applications (Rahman et al., 2002; Tyagi et al., 2011).

- From the current study as well as some other studies, the introduced strain population was found to decrease shortly after being inoculated due to several abiotic and biotic stresses. Repeated inoculation is required to maintain the bioaugmentation efficiency. To improve the bioaugmentation efficiency, immobilization of specialized consortia has been reported as a strategy for maintaining efficient degradation in wastewater treatment (Bouchez et al., 2009; El-Naas et al., 2009; Qu et al., 2006). However, immobilization of degrading strains within alginate beads or polyvinyl alcohol etc. may be costly and complex for practical application. To overcome this problem, immobilization of specialized degraders into biofilms reactors, e.g. Moving Bed Biofilm Reactor (MBBR), or biological contact oxidation reactor (BCOR) or membrane-aerated-biofilm reactors (MABR) etc., has been found to enhance the degradation of toxic pollutants in wastewater (Fang et al., 2013; Li et al., 2013; Wang et al., 2012). It is proposed that immobilization of the specialized degraders onto biofilm carriers in a membrane bioreactor (biofilm membrane bioreactor-BF-MBR) could potentially enhance the survival, prevent wash-out and out-competition of newly introduced strains in bioaugmented systems, thus improving the bioaugmentation efficiency. In addition, the potential ecotoxicological effects of the transformation metabolites present in the effluent should also be monitored.
- In addition to monitoring the abundance, microbial monitoring tools, e.g. primer designs, qPCR, etc., could also be developed to target the SMX antibiotic resistance genes of the inoculated strain *A. denitrificans* PR1 in order to monitor the resistance levels in both the bioaugmented and non-bioaugmented reactors. Sequencing of the 16S by Next Generation Sequencing can also be used to understand the dynamic of the activated sludge community.
- In this study, heterotrophs seem to be the dominant organisms responsible for the biotransformation of SMX. Hence, the calibration of cometabolic model based on only organic carbon as the primary substrates. But both autotrophic (Kassotaki et al., 2016) and heterotrophic (Müller et al., 2013) bacteria can be responsible for the removal of SMX. Further research could be extended to combining modeling and experimental efforts to understand the contribution of different members, e.g. autotrophs, in a community in terms of SMX and other MP biodegradations.
- Bioaugmentation is a promising strategy for xenobiotics bioremediation, but not yet widely applied due to its lower predictability and controllability. Bioaugmentation success requires

not only engineering aspects, but also a deep understanding of microbial ecology. Technological advances in molecular microbial ecology together with analytical chemistry provide us more direct, comprehensive assessment of microbial community structure and composition (Thompson et al., 2005). Bioaugmentation is considered as a specific application of co-cultures and represents a kind of microbial invasion process. Mathematical modelling is becoming more and more appreciated as a tool for identifying possible co-cultures of interest, and most recently, Individual-Based Modelling has been developed and able to retrieve in vitro dynamics of invasion in bioaugmented sand filter communities (Daly et al., 2018). In addition, support vector regression models using microbial community information and operational data were proposed to reliably predict the reactor performance of bioaugmentation systems (Seshan et al., 2014). Combination of these new technologies and models calls for further studies to better understand the interactions between the bioaugmented degrader and the resident community. This ultimately allows bioaugmentation to be more predictable and controllable upon changes in systems.

REFERENCES

Abegglen, C., Joss, A., McArdell, C.S., Fink, G., Schlüsener, M.P., Ternes, T.A., Siegrist, H., 2009. The fate of selected micropollutants in a single-house MBR. Water Res. 43, 2036–2046. https://doi.org/10.1016/j.watres.2009.02.005

Akhtar, J., Amin, N.S., Aris, A., 2011. Combined adsorption and catalytic ozonation for removal of sulfamethoxazole using Fe2O3/CeO2 loaded activated carbon. Chem. Eng. J. 170, 136–144. https://doi.org/http://dx.doi.org/10.1016/j.cej.2011.03.043

Al-Ahmad, A., Daschner, F.D., Kümmerer, K., 1999. Biodegradability of Cefotiam, Ciprofloxacin, Meropenem, Penicillin G, and Sulfamethoxazole and Inhibition of Waste Water Bacteria. Arch. Environ. Contam. Toxicol. 37, 158–163. https://doi.org/10.1007/s002449900501

Alexy, R., Kümpel, T., Kümmerer, K., 2004. Assessment of degradation of 18 antibiotics in the Closed Bottle Test. Chemosphere 57, 505–512. https://doi.org/https://doi.org/10.1016/j.chemosphere.2004.06.024

Alidina, M., Li, D., Ouf, M., Drewes, J.E., 2014. Role of primary substrate composition and concentration on attenuation of trace organic chemicals in managed aquifer recharge systems. J. Environ. Manage. 144, 58–66. https://doi.org/https://doi.org/10.1016/j.jenvman.2014.04.032

Altenburger, R., Walter, H., Grote, M., 2004. What Contributes to the Combined Effect of a Complex Mixture? Environ. Sci. Technol. 38, 6353–6362. https://doi.org/10.1021/es049528k

Alvarez-Cohen, L., Speitel, G.E., 2001. Kinetics of aerobic cometabolism of chlorinated solvents. Biodegradation 12, 105–126. https://doi.org/10.1023/A:1012075322466

Alvarino, T., Nastold, P., Suarez, S., Omil, F., Corvini, P.F.X., Bouju, H., 2016. Role of biotransformation, sorption and mineralization of 14C-labelled sulfamethoxazole under different redox conditions. Sci. Total Environ. 542, Part, 706–715. https://doi.org/https://doi.org/10.1016/j.scitotenv.2015.10.140

Andersen, H.R., Hansen, M., Kjølholt, J., Stuer-Lauridsen, F., Ternes, T., Halling-Sørensen, B., 2005. Assessment of the importance of sorption for steroid estrogens removal during activated sludge treatment. Chemosphere 61, 139–146. https://doi.org/https://doi.org/10.1016/j.chemosphere.2005.02.088

Andreozzi, R., Cesaro, R., Marotta, R., Pirozzi, F., 2006. Evaluation of biodegradation kinetic constants for aromatic compounds by means of aerobic batch experiments. Chemosphere 62, 1431–1436. https://doi.org/https://doi.org/10.1016/j.chemosphere.2005.05.041

Arp, D.J., Yeager, C.M., Hyman, M.R., 2001. Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene. Biodegradation 12, 81–103. https://doi.org/10.1023/A:1012089908518

Ashton, D., Hilton, M., Thomas, K. V, 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. Sci. Total Environ. 333, 167–184. https://doi.org/https://doi.org/10.1016/j.scitotenv.2004.04.062

Barbosa, M.O., Moreira, N.F.F., Ribeiro, A.R., Pereira, M.F.R., Silva, A.M.T., 2016. Occurrence and removal of organic micropollutants: An overview of the watch list of EU Decision 2015/495. Water Res. 94, 257–279. https://doi.org/https://doi.org/10.1016/j.watres.2016.02.047

Barbot, E., Seyssiecq, I., Roche, N., Marrot, B., 2010. Inhibition of activated sludge respiration by sodium azide addition: Effect on rheology and oxygen transfer. Chem. Eng. J. 163, 230–235. https://doi.org/10.1016/j.cej.2010.07.050

Barreiros, L., Nogales, B., Manaia, C.M., Ferreira, A.C.S., Pieper, D.H., Reis, M.A., Nunes, O.C., 2003. A novel pathway for mineralization of the thiocarbamate herbicide molinate by a defined bacterial mixed culture. Environ. Microbiol. 5, 944–953. https://doi.org/10.1046/j.1462-

2920.2003.00492.x

Batt, A.L., Bruce, I.B., Aga, D.S., 2006a. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Environ. Pollut. 142, 295–302. https://doi.org/10.1016/J.ENVPOL.2005.10.010

Batt, A.L., Kim, Sungpyo, A., Diana S., A., 2006b. Enhanced Biodegradation of Iopromide and Trimethoprim in Nitrifying Activated Sludge†. https://doi.org/10.1021/ES060835V

Batt, A.L., Kim, S., Aga, D.S., 2007. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. Chemosphere 68, 428–435. https://doi.org/http://dx.doi.org/10.1016/j.chemosphere.2007.01.008

Behera, S.K., Kim, H.W., Oh, J.E., Park, H.S., 2011. Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. Sci. Total Environ. 409, 4351–4360. https://doi.org/10.1016/j.scitotenv.2011.07.015

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439

Bertelkamp, C., Verliefde, A.R.D., Reynisson, J., Singhal, N., Cabo, A.J., de Jonge, M., van der Hoek, J.P., 2016. A predictive multi-linear regression model for organic micropollutants, based on a laboratory-scale column study simulating the river bank filtration process. J. Hazard. Mater. 304, 502–511. https://doi.org/https://doi.org/10.1016/j.jhazmat.2015.11.003

Boon, N., De Gelder, L., Lievens, H., Siciliano, S.D., Top, E.M., Verstraete, W., 2002. Bioaugmenting bioreactors for the continuous removal of 3-chloroaniline by a slow release approach. Environ. Sci. Technol. 36, 4698–4704. https://doi.org/10.1021/es020076q

Boon, N., Goris, J., Vos, P. De, Verstraete, W., Top, E.M., 2000. Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Comamonas testosteroni Strain , I2 gfp. Appl. Environ. Microbiol. 66, 2906–13. https://doi.org/10.1128/AEM.66.7.2906-2913.2000.Updated

Boon, N., Top, E.M., Verstraete, W., Siciliano, S.D., 2003. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. Appl. Environ. Microbiol. 69, 1511–1520. https://doi.org/10.1128/AEM.69.3.1511-1520.2003

Boon, N., Verstraete, W., 2010. Bioaugmentation of Hydrocarbons, in: Timmis, K. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, pp. 2531–2543. https://doi.org/10.1007/978-3-540-77587-4_188

Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Doré, J., Delgenès, P., Moletta, R., Wagner, M., 2000. Ecological study of a bioaugmentation failure. Environ. Microbiol. 2, 179–190. https://doi.org/10.1046/j.1462-2920.2000.00091.x

Bouchez, T., Patureau, D., Delgenès, J.P., Moletta, R., 2009. Successful bacterial incorporation into activated sludge flocs using alginate. Bioresour. Technol. 100, 1031–1032. https://doi.org/http://dx.doi.org/10.1016/j.biortech.2008.07.028

Bouju, H., Ricken, B., Beffa, T., Corvini, P.F.X., Kolvenbach, B.A., 2012. Isolation of Bacterial Strains Capable of Sulfamethoxazole Mineralization from an Acclimated Membrane Bioreactor. Appl. Environ. Microbiol. 78, 277–279. https://doi.org/10.1128/aem.05888-11

Bowman, J.C., Zhou, J.L., Readman, J.W., 2002. Sediment–water interactions of natural oestrogens under estuarine conditions. Mar. Chem. 77, 263–276. https://doi.org/https://doi.org/10.1016/S0304-4203(02)00006-3

Boxall, A.B.A., Fogg, L.A., Blackwell, P.A., Blackwell, P., Kay, P., Pemberton, E.J., Croxford, A., 2004. Veterinary Medicines in the Environment, in: Reviews of Environmental Contamination and Toxicology. Springer New York, New York, NY, pp. 1–91. https://doi.org/10.1007/0-387-21729-0_1

Brian, J. V, Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfà, A., Marcomini, A., Sumpter, J.P., 2005. Accurate Prediction of the Response of Freshwater Fish to a Mixture of Estrogenic Chemicals. Environ. Health Perspect. 113, 721–728. https://doi.org/10.1289/ehp.7598

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy, effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci. Total Environ. 366, 772–783. https://doi.org/10.1016/j.scitotenv.2005.10.007

Carballa, M., Fink, G., Omil, F., Lema, J.M., Ternes, T., 2008. Determination of the solid-water distribution coefficient (Kd) for pharmaceuticals, estrogens and musk fragrances in digested sludge. Water Res. 42, 287–295. https://doi.org/10.1016/j.watres.2007.07.012

Carballa, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M., Ternes, T., 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Res. 38, 2918–2926. https://doi.org/http://dx.doi.org/10.1016/j.watres.2004.03.029

Chang, H.L., Alvarez-Cohen, L., 1995. Model for the Cometabolic Biodegradation of Chlorinated Organics. Environ. Sci. Technol. 29, 2357–2367. https://doi.org/Doi 10.1021/Es00009a031

Chen, J.N., Zhan, P., Koopman, B., Fang, G.G., Shi, Y.Q., 2012. Bioaugmentation with Gordonia strain JW8 in treatment of pulp and paper wastewater. Clean Technol. Environ. Policy 14, 899–904. https://doi.org/10.1007/s10098-012-0459-4

Chèvre, N., Loepfe, C., Singer, H., Stamm, C., Fenner, K., Escher, B.I., 2006. Including Mixtures in the Determination of Water Quality Criteria for Herbicides in Surface Water. Environ. Sci. Technol. 40, 426–435. https://doi.org/10.1021/es050239l

Chong, N.M., Chiou, J.N., 2010. Biodegradation of BTE in soil by indigenous microbial populations with and without biogenic substrates. World J. Microbiol. Biotechnol. 26, 1587–1594. https://doi.org/10.1007/s11274-010-0334-6

Chong, N.M., Luong, M.L., Hwu, C.S., 2012. Biogenic substrate benefits activated sludge in acclimation to a xenobiotic. Bioresour. Technol. 104, 181–186. https://doi.org/10.1016/j.biortech.2011.11.004

Cirja, M., Ivashechkin, P., Schäffer, A., Corvini, P.F.X., 2008. Factors affecting the removal of organic micropollutants from wastewater in conventional treatment plants (CTP) and membrane bioreactors (MBR). Rev. Environ. Sci. Biotechnol. 7, 61–78. https://doi.org/10.1007/s11157-007-9121-8

Clara, M., Kreuzinger, N., Strenn, B., Gans, O., Kroiss, H., 2005a. The solids retention time—a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. Water Res. 39, 97–106. https://doi.org/http://dx.doi.org/10.1016/j.watres.2004.08.036

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H., 2005b. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Res. 39, 4797–4807. https://doi.org/http://dx.doi.org/10.1016/j.watres.2005.09.015

Clouzot, L., Choubert, J.-M., Cloutier, F., Goel, R., Love, N.G., Melcer, H., Ort, C., Patureau, D., Plósz, B.G., Pomiès, M., Vanrolleghem, P.A., 2013. Perspectives on modelling micropollutants in wastewater treatment plants. Water Sci. Technol. 68, 448–61.

https://doi.org/10.2166/wst.2013.272

Collado, N., Buttiglieri, G., Marti, E., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Comas, J., Rodriguez-Roda, I., 2013. Effects on activated sludge bacterial community exposed to sulfamethoxazole. Chemosphere 93, 99–106. https://doi.org/http://dx.doi.org/10.1016/j.chemosphere.2013.04.094

Cossins Bowler, K., A.R., 1987. Temperature Biology of Animals. Chapman & Hall, London.

Costanzo, S.D., Murby, J., Bates, J., 2005. Ecosystem response to antibiotics entering the aquatic environment. Mar. Pollut. Bull. 51, 218–223. https://doi.org/10.1016/j.marpolbul.2004.10.038

Criddle, C.S., 1993. The kinetics of cometabolism. Biotechnol. Bioeng. 41, 1048–56. https://doi.org/10.1002/bit.260411107

Dabert, P., Delgenès, J.-P., Moletta, R., Godon, J.-J., 2002. Contribution of molecular microbiology to the study in water pollution removal of microbial community dynamics. Rev. Environ. Sci. Biotechnol. 1, 39–49. https://doi.org/10.1023/A:1015127411408

Dalton, H., Stirling, D.I., 1982. Co-metabolism. Philos. Trans. R. Soc. London Ser. B-Biological Sci. 297, 481–496. https://doi.org/10.1098/rstb.1982.0056

Daly, A., Baetens, J., Vandermaesen, J., Boon, N., Springael, D., De Baets, B., 2018. Individual-Based Modelling of Invasion in Bioaugmented Sand Filter Communities. Processes 6, 2.

Dantas, G., Sommer, M.O.A., Oluwasegun, R.D., Church, G.M., 2008. Bacteria Subsisting on Antibiotics. Science (80-.). 320, 100–103. https://doi.org/10.1126/science.1155157

De Gusseme, B., Vanhaecke, L., Verstraete, W., Boon, N., 2011. Degradation of acetaminophen by Delftia tsuruhatensis and Pseudomonas aeruginosa in a membrane bioreactor. Water Res. 45, 1829–1837. https://doi.org/http://dx.doi.org/10.1016/j.watres.2010.11.040

de Lipthay, J.R., Sørensen, S.R., Aamand, J., 2007. Effect of herbicide concentration and organic and inorganic nutrient amendment on the mineralization of mecoprop, 2,4-D and 2,4,5-T in soil and aquifer samples. Environ. Pollut. 148, 83–93. https://doi.org/10.1016/j.envpol.2006.11.005

de Voogt, P., Janex-Habibi, M.-L., Sacher, F., Puijker, L., Mons, M., 2009. Development of a common priority list of pharmaceuticals relevant for the water cycle. Water Sci. Technol. 59, 39–46. https://doi.org/10.2166/wst.2009.764

Drewes, J.E., 2007. Chapter 4.1 Removal of pharmaceutical residues during wastewater treatment, in: Petrović, M., Barceló, D. (Eds.), Comprehensive Analytical Chemistry. Elsevier, pp. 427–449. https://doi.org/http://dx.doi.org/10.1016/S0166-526X(07)50013-9

Drillia, P., Dokianakis, S.N., Fountoulakis, M.S., Kornaros, M., Stamatelatou, K., Lyberatos, G., 2005a. On the occasional biodegradation of pharmaceuticals in the activated sludge process: The example of the antibiotic sulfamethoxazole. J. Hazard. Mater. 122, 259–265. https://doi.org/10.1016/J.JHAZMAT.2005.03.009

Drillia, P., Stamatelatou, K., Lyberatos, G., 2005b. Fate and mobility of pharmaceuticals in solid matrices. Chemosphere 60, 1034–1044. https://doi.org/10.1016/J.CHEMOSPHERE.2005.01.032

Duque, A.F., Bessa, V.S., Carvalho, M.F., Castro, P.M.L., 2011. Bioaugmentation of a rotating biological contactor for degradation of 2-fluorophenol. Bioresour. Technol. 102, 9300–9303. https://doi.org/10.1016/j.biortech.2011.07.003

Dytczak, M.A., Londry, K.L., Oleszkiewicz, J.A., 2008. Biotransformation of estrogens in nitrifying activated sludge under aerobic and alternating anoxic/aerobic conditions. Water Environ. Res. 80, 47–52. https://doi.org/10.2175/106143007x184690

ECDC, 2014. Summary of the latest data on antibiotic consumption in EU: 2014. European Centre for Disease Prevention and Control.

Egli, T., 2010. How to live at very low substrate concentration. Water Res. 44, 4826–4837. https://doi.org/10.1016/j.watres.2010.07.023

Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y.S., Goto, K., Hirata, K., Miyamoto, K., Yoshimura, H., 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57, 1733–1738. https://doi.org/10.1016/j.chemosphere.2004.07.017

El-Naas, M.H., Al-Muhtaseb, S.A., Makhlouf, S., 2009. Biodegradation of phenol by Pseudomonas putida immobilized in polyvinyl alcohol (PVA) gel. J. Hazard. Mater. 164, 720–725. https://doi.org/http://dx.doi.org/10.1016/j.jhazmat.2008.08.059

Ellis, D.E., Lutz, E.J., Odom, J.M., Buchanan, R.J., Bartlett, C.L., Lee, M.D., Harkness, M.R., DeWeerd, K.A., 2000. Bioaugmentation for Accelerated In Situ Anaerobic Bioremediation. Environ. Sci. Technol. 34, 2254–2260. https://doi.org/10.1021/es990638e

Escher, B.I., Baumgartner, R., Koller, M., Treyer, K., Lienert, J., McArdell, C.S., 2011. Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. Water Res. 45, 75–92. https://doi.org/http://dx.doi.org/10.1016/j.watres.2010.08.019

Falås, P., Baillon-Dhumez, A., Andersen, H.R., Ledin, A., La Cour Jansen, J., 2012. Suspended biofilm carrier and activated sludge removal of acidic pharmaceuticals. Water Res. 46, 1167–1175. https://doi.org/10.1016/j.watres.2011.12.003

Falås, P., Longrée, P., La Cour Jansen, J., Siegrist, H., Hollender, J., Joss, A., 2013. Micropollutant removal by attached and suspended growth in a hybrid biofilm-activated sludge process. Water Res. 47, 4498–4506. https://doi.org/10.1016/j.watres.2013.05.010

Falas, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T.A., Joss, A., 2016. Tracing the limits of organic micropollutant removal in biological wastewater treatment. Water Res. 95, 240–249. https://doi.org/10.1016/j.watres.2016.03.009

Fang, F., Han, H., Zhao, Q., Xu, C., Zhang, L., 2013. Bioaugmentation of biological contact oxidation reactor (BCOR) with phenol-degrading bacteria for coal gasification wastewater (CGW) treatment. Bioresour. Technol. 150, 314–320. https://doi.org/http://dx.doi.org/10.1016/j.biortech.2013.09.119

Fatta-Kassinos, D., Bester, K., Kümmerer, K. (Eds.), 2010. Xenobiotics in the Urban Water Cycle, Environmental Pollution. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-90-481-3509-7

Fatta-Kassinos, D., Vasquez, M.I., Kümmerer, K., 2011. Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes – Degradation, elucidation of byproducts and assessment of their biological potency. Chemosphere 85, 693–709. https://doi.org/https://doi.org/10.1016/j.chemosphere.2011.06.082

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159. https://doi.org/http://dx.doi.org/10.1016/j.aquatox.2005.09.009

Fenu, A., Donckels, B.M.R., Beffa, T., Bemfohr, C., Weemaes, M., 2015. Evaluating the application of Microbacterium sp. strain BR1 for the removal of sulfamethoxazole in full-scale membrane bioreactors. Water Sci. Technol. 72, 1754–1761. https://doi.org/10.2166/wst.2015.397

Fernandez-Fontaina, E., Omil, F., Lema, J.M., Carballa, M., 2012. Influence of nitrifying conditions on the biodegradation and sorption of emerging micropollutants. Water Res. 46, 5434–5444. https://doi.org/10.1016/J.WATRES.2012.07.037

Flaherty, C.M., Dodson, S.I., 2005. Effects of pharmaceuticals on Daphnia survival, growth, and reproduction. Chemosphere 61, 200–207. https://doi.org/https://doi.org/10.1016/j.chemosphere.2005.02.016

Frascari, D., Cappelletti, M., Fedi, S., Zannoni, D., Nocentini, M., Pinelli, D., 2010. 1,1,2,2-

Tetrachloroethane aerobic cometabolic biodegradation in slurry and soil-free bioreactors: A kinetic study. Biochem. Eng. J. 52, 55–64. https://doi.org/http://dx.doi.org/10.1016/j.bej.2010.07.004

Gao, P., Ding, Y., Li, H., Xagoraraki, I., 2012. Occurrence of pharmaceuticals in a municipal wastewater treatment plant: Mass balance and removal processes. Chemosphere 88, 17–24. https://doi.org/10.1016/J.CHEMOSPHERE.2012.02.017

Gao, P., Mao, D., Luo, Y., Wang, L., Xu, B., Xu, L., 2012. Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. Water Res. 46, 2355–2364. https://doi.org/https://doi.org/10.1016/j.watres.2012.02.004

García-Galán, M.J., Frömel, T., Müller, J., Peschka, M., Knepper, T., Díaz-Cruz, S., Barceló, D., 2012. Biodegradation studies of N 4-acetylsulfapyridine and N 4-acetylsulfamethazine in environmental water by applying mass spectrometry techniques. Anal. Bioanal. Chem. 402, 2885–2896. https://doi.org/10.1007/s00216-012-5751-y

Gaulke, L.S., Strand, S.E., Kalhorn, T.F., Stensel, H.D., 2009. Estrogen Biodegradation Kinetics and Estrogenic Activity Reduction for Two Biological Wastewater Treatment Methods. Environ. Sci. Technol. 43, 7111–7116. https://doi.org/10.1021/es901194c

Gaulke, L.S., Strand, S.E., Kalhorn, T.F., Stensel, H.D., 2008. 17α-ethinylestradiol Transformation via Abiotic Nitration in the Presence of Ammonia Oxidizing Bacteria. Environ. Sci. Technol. 42, 7622–7627. https://doi.org/10.1021/es801503u

Gauthier, H., Yargeau, V., Cooper, D.G., 2010. Biodegradation of pharmaceuticals by Rhodococcus rhodochrous and Aspergillus niger by co-metabolism. Sci. Total Environ. 408, 1701–1706. https://doi.org/http://dx.doi.org/10.1016/j.scitotenv.2009.12.012

Gentry, T., Rensing, C., Pepper, I., 2004. New Approaches for Bioaugmentation as a Remediation Technology. Crit. Rev. Environ. Sci. Technol. 34, 447–494. https://doi.org/10.1080/10643380490452362

Göbel, A., McArdell, C.S., Joss, A., Siegrist, H., Giger, W., 2007. Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Sci. Total Environ. 372, 361–371. https://doi.org/10.1016/j.scitotenv.2006.07.039

Göbel, A., Thomsen, A., Christa S. McArdell, *, Adriano Joss, A., Giger, W., 2005. Occurrence and Sorption Behavior of Sulfonamides, Macrolides, and Trimethoprim in Activated Sludge Treatment. https://doi.org/10.1021/ES048550A

Goldstein, R.M., Mallory, L.M., Alexander, M., 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. 50, 977–983.

Gonzalez-Pleiter, M., Gonzalo, S., Rodea-Palomares, I., Leganes, F., Rosal, R., Boltes, K., Marco, E., Fernandez-Pinas, F., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment. Water Res. 47, 2050–2064. https://doi.org/10.1016/j.watres.2013.01.020

Gothwal, R., Shashidhar, T., 2015. Antibiotic Pollution in the Environment: A Review. Clean - Soil, Air, Water 43, 479–489. https://doi.org/10.1002/clen.201300989

Gros, M., Petrović, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environ. Int. 36, 15–26. https://doi.org/http://dx.doi.org/10.1016/j.envint.2009.09.002

Gujer, W., Henze, M., Mino, T., Matsuo, T., Wentzel, M.C., Marais v., G.R., 1995. The activated sludge model No. 2: Biological phosphorus removal. Water Sci. Technol. 31, 1–11. https://doi.org/10.1016/0273-1223(95)00175-M

Hai, F.I., Tessmer, K., Nguyen, L.N., Kang, J., Price, W.E., Nghiem, L.D., 2011. Removal of

micropollutants by membrane bioreactor under temperature variation. J. Memb. Sci. 383, 144–151. https://doi.org/https://doi.org/10.1016/j.memsci.2011.08.047

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment- A review. Chemosphere 36, 357–393. https://doi.org/http://dx.doi.org/10.1016/S0045-6535(97)00354-8

Hamon, P., Villain, M., Marrot, B., 2014. Determination of sorption properties of micropollutants: What is the most suitable activated sludge inhibition technique to preserve the biomass structure? Chem. Eng. J. 242, 260–268. https://doi.org/10.1016/J.CEJ.2013.07.117

Han, L., Wang, J., Shi, H., Qian, Y., 2000. Bioaugmenation: a new strategy for removal of recalcitrant compounds in wastewater - a case study of quinoline. J. Environ. Sci. 12, 22–25.

Hashimoto, T., Onda, K., Morita, T., Luxmy, B.S., Tada, K., Miya, A., Murakami, T., 2010. Contribution of the Estrogen-Degrading Bacterium *Novosphingobium* sp. Strain JEM-1 to Estrogen Removal in Wastewater Treatment. J. Environ. Eng. 136, 890–896. https://doi.org/doi:10.1061/(ASCE)EE.1943-7870.0000218

Helbling, D.E., Ackermann, M., Fenner, K., Kohler, H.-P.E., Johnson, D.R., 2012. The activity level of a microbial community function can be predicted from its metatranscriptome. ISME J. 6, 902–4. https://doi.org/10.1038/ismej.2011.158

Helbling, D.E., Johnson, D.R., Honti, M., Fenner, K., 2012. Micropollutant Biotransformation Kinetics Associate with WWTP Process Parameters and Microbial Community Characteristics. Environ. Sci. Technol. 46, 10579–10588. https://doi.org/10.1021/es3019012

Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G. v R., Van Loosdrecht, M.C.M., 1999. Activated sludge model No.2D, ASM2D. Water Sci. Technol. 39, 165–182. https://doi.org/https://doi.org/10.1016/S0273-1223(98)00829-4

Henze, M., Gujer, W., Mino, T., van Loosdrecht, M.C.M., 2000. Activated Sludge Models ASM1, ASM2, ASM2d and ASM3. IWA Publ. 121. https://doi.org/10.1007/s13398-014-0173-7.2

Henze, M., Jr, C.P.L.G., Gujer, W., Marais, G.V.R., Matsuo, T., 1987. A general model for single-sludge wastewater treatment systems. Water Res. 21, 505–515. https://doi.org/http://dx.doi.org/10.1016/0043-1354(87)90058-3

Herrero, M., Stuckey, D.C., 2015. Bioaugmentation and its application in wastewater treatment: A review. Chemosphere 140, 119–128. https://doi.org/10.1016/j.chemosphere.2014.10.033

Herzog, B., Lemmer, H., Horn, H., Müller, E., 2013. Characterization of pure cultures isolated from sulfamethoxazole-acclimated activated sludge with respect to taxonomic identification and sulfamethoxazole biodegradation potential. BMC Microbiol. 13, 276. https://doi.org/10.1186/1471-2180-13-276

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.-L., 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225, 109–118. https://doi.org/http://dx.doi.org/10.1016/S0048-9697(98)00337-4

Hoeger, B., Köllner, B., Dietrich, D.R., Hitzfeld, B., 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f. fario). Aquat. Toxicol. 75, 53–64. https://doi.org/https://doi.org/10.1016/j.aquatox.2005.07.006

Hörsing, M., Ledin, A., Grabic, R., Fick, J., Tysklind, M., Jansen, J. la C., Andersen, H.R., 2011. Determination of sorption of seventy-five pharmaceuticals in sewage sludge. Water Res. 45, 4470–4482. https://doi.org/http://dx.doi.org/10.1016/j.watres.2011.05.033

Hyland, K.C., Dickenson, E.R. V, Drewes, J.E., Higgins, C.P., 2012. Sorption of ionized and neutral emerging trace organic compounds onto activated sludge from different wastewater treatment

configurations. Water Res. 46, 1958–1968. https://doi.org/10.1016/j.watres.2012.01.012

Iasur-Kruh, L., Hadar, Y., Minz, D., 2011. Isolation and Bioaugmentation of an Estradiol-Degrading Bacterium and Its Integration into a Mature Biofilm. Appl. Environ. Microbiol. 77, 3734–3740. https://doi.org/10.1128/aem.00691-11

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci. Total Environ. 346, 87–98. https://doi.org/10.1016/j.scitotenv.2004.11.017

Jacob J., F.. M., 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3, 318–356.

Jiang, B., Li, A., Cui, D., Cai, R., Ma, F., Wang, Y., 2014. Biodegradation and metabolic pathway of sulfamethoxazole by Pseudomonas psychrophila HA-4, a newly isolated cold-adapted sulfamethoxazole-degrading bacterium. Appl. Microbiol. Biotechnol. 98, 4671–4681. https://doi.org/10.1007/s00253-013-5488-3

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506. https://doi.org/10.1021/es9710870

Joss, A., Andersen, H., Ternes, T., Richle, P.R., Siegrist, H., 2004. Removal of estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: Consequences for plant optimization. Environ. Sci. Technol. 38, 3047–3055. https://doi.org/10.1021/es0351488

Joss, A., Carballa, M., Kreuzinger, N., Siegrist, H., Zabczynski, S., 2006a. Wastewater treatment, in: Joss Ternes, T.A. (eds.), A. (Ed.), "Human Pharmaceuticals, Hormones and Fragrances—The Challenge of Micropollutants in Urban Water Management." IWA Publishing, London, UK.

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 Res. 39, 3139–3152. https://doi.org/10.1016/j.watres.2005.05.031

Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C.S., Ternes, T.A., Thomsen, A., Siegrist, H., 2006b. Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. Water Res. 40, 1686–1696. https://doi.org/10.1016/j.watres.2006.02.014

Karickhoff, S.W., Brown, D.S., Scott, T.A., 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13, 241–248. https://doi.org/https://doi.org/10.1016/0043-1354(79)90201-X

Kassotaki, E., Buttiglieri, G., Ferrando-Climent, L., Rodriguez-Roda, I., Pijuan, M., 2016. Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products. Water Res. 94, 111–119. https://doi.org/http://dx.doi.org/10.1016/j.watres.2016.02.022

Kerry, J., Slattery, M., Vaughan, S., Smith, P., 1996. The importance of bacterial multiplication in the selection, by oxytetracycline-HCl, of oxytetracycline-resistant bacteria in marine sediment microcosms. Aquaculture 144, 103–119. https://doi.org/10.1016/s0044-8486(96)01321-x

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 17??-ethinylestradiol and trimethoprim. Environ. Sci. Technol. 45, 3605–3612. https://doi.org/10.1021/es1037035

Kim, J.W., Jang, H.S., Kim, J.G., Ishibashi, H., Hirano, M., Nasu, K., Ichikawa, N., Takao, Y., Shinohara, R., Arizono, K., 2009. Occurrence of Pharmaceutical and Personal Care Products (PPCPs) in Surface Water from Mankyung River, South Korea. J. Heal. Sci. 55, 249–258.

Kimura, K., Hara, H., Watanabe, Y., 2007. Elimination of selected acidic pharmaceuticals from

municipal wastewater by an activated sludge system and membrane bioreactors. Environ. Sci. Technol. 41, 3708–3714. https://doi.org/10.1021/es061684z

Kimura, K., Hara, H., Watanabe, Y., 2005. Removal of pharmaceutical compounds by submerged membrane bioreactors (MBRs). Desalination 178, 135–140. https://doi.org/10.1016/j.desal.2004.11.033

Kimura, K., Toshima, S., Amy, G., Watanabe, Y., 2004. Rejection of neutral endocrine disrupting compounds (EDCs) and pharmaceutical active compounds (PhACs) by RO membranes. J. Memb. Sci. 245, 71–78. https://doi.org/http://dx.doi.org/10.1016/j.memsci.2004.07.018

Knopp, G., Prasse, C., Ternes, T.A., Cornel, P., 2016. Elimination of micropollutants and transformation products from a wastewater treatment plant effluent through pilot scale ozonation followed by various activated carbon and biological filters. Water Res. 100, 580–592. https://doi.org/https://doi.org/10.1016/j.watres.2016.04.069

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. https://doi.org/10.1021/ES011055J

Kolvenbach, B.A., Helbling, D.E., Kohler, H.P.E., Corvini, P.F.X., 2014. Emerging chemicals and the evolution of biodegradation capacities and pathways in bacteria. Curr. Opin. Biotechnol. 27, 8–14. https://doi.org/10.1016/j.copbio.2013.08.017

Kong, W.D., Zhu, Y.G., Liang, Y.C., Zhang, J., Smith, F.A., Yang, A., 2007. Uptake of oxytetracycline and its phytotoxicity to alfalfa (Medicago sativa L.). Environ. Pollut. 147, 187–193. https://doi.org/10.1016/j.envpol.2006.08.016

Kovalova, L., Siegrist, H., Singer, H., Wittmer, A., McArdell, C.S., 2012. Hospital wastewater treatment by membrane bioreactor: Performance and efficiency for organic micropollutant elimination. Environ. Sci. Technol. 46, 1536–1545. https://doi.org/10.1021/es203495d

Kumar, A., Xagoraraki, I., 2010. Pharmaceuticals, personal care products and endocrine-disrupting chemicals in U.S. surface and finished drinking waters: A proposed ranking system. Sci. Total Environ. 408, 5972–5989. https://doi.org/http://dx.doi.org/10.1016/j.scitotenv.2010.08.048

Kumar, V., Johnson, A.C., Nakada, N., Yamashita, N., Tanaka, H., 2012. De-conjugation behavior of conjugated estrogens in the raw sewage, activated sludge and river water. J. Hazard. Mater. 227–228, 49–54. https://doi.org/10.1016/J.JHAZMAT.2012.04.078

Lai, H.-T., Hou, J.-H., Su, C.-I., Chen, C.-L., 2009. Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae Chlorella pyrenoidosa, Isochrysis galbana, and Tetraselmis chui. Ecotoxicol. Environ. Saf. 72, 329–334. https://doi.org/https://doi.org/10.1016/j.ecoenv.2008.03.005

Larcher, S., Yargeau, V., 2011. Biodegradation of sulfamethoxazole by individual and mixed bacteria. Appl. Microbiol. Biotechnol. 91, 211–218. https://doi.org/10.1007/s00253-011-3257-8

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Gaze, W.H., 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ. Int. 82, 92–100. https://doi.org/https://doi.org/10.1016/j.envint.2015.02.013

Li, B., Zhang, T., 2011. Mass flows and removal of antibiotics in two municipal wastewater treatment plants. Chemosphere 83, 1284–1289. https://doi.org/10.1016/j.chemosphere.2011.03.002

Li, B., Zhang, T., 2010. Biodegradation and Adsorption of Antibiotics in the Activated Sludge Process. Environ. Sci. Technol. 44, 3468–3473. https://doi.org/10.1021/es903490h

Li, C., Li, Y., Cheng, X., Feng, L., Xi, C., Zhang, Y., 2013. Immobilization of Rhodococcus rhodochrous BX2 (an acetonitrile-degrading bacterium) with biofilm-forming bacteria for wastewater treatment. Bioresour. Technol. 131, 390–396.

https://doi.org/http://dx.doi.org/10.1016/j.biortech.2012.12.140

Li, D., Alidina, M., Drewes, J.E., 2014. Role of primary substrate composition on microbial community structure and function and trace organic chemical attenuation in managed aquifer recharge systems. Appl. Microbiol. Biotechnol. 98, 5747–5756. https://doi.org/10.1007/s00253-014-5677-8

Li, F., Desmiarti, R., Yuasa, A., Horio, A., 2008. Behavior of natural estrogens in semicontinuous activated sludge biodegradation reactors. Bioresour. Technol. 99, 2964–2971. https://doi.org/10.1016/J.BIORTECH.2007.06.016

Li, W.C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. Environ. Pollut. 187, 193–201. https://doi.org/http://dx.doi.org/10.1016/j.envpol.2014.01.015

Lindblom, E., Press-Kristensen, K., Vanrolleghem, P.A., Mikkelsen, P.S., Henze, M., 2009. Dynamic experiments with high bisphenol-A concentrations modelled with an ASM model extended to include a separate XOC degrading microorganism. Water Res. 43, 3169–3176. https://doi.org/10.1016/j.watres.2009.04.030

Lindsey, M.E., Meyer, M., Thurman, E.M., 2001. Analysis of Trace Levels of Sulfonamide and Tetracycline Antimicrobials in Groundwater and Surface Water Using Solid-Phase Extraction and Liquid Chromatography/Mass Spectrometry. October 73, 4640–4646.

Liu, C., Huang, X., Wang, H., 2008. Start-up of a membrane bioreactor bioaugmented with genetically engineered microorganism for enhanced treatment of atrazine containing wastewater. Desalination 231, 12–19. https://doi.org/10.1016/j.desal.2007.11.034

Liu, P., Zhang, H., Feng, Y., Yang, F., Zhang, J., 2014. Removal of trace antibiotics from wastewater: A systematic study of nanofiltration combined with ozone-based advanced oxidation processes. Chem. Eng. J. 240, 211–220. https://doi.org/https://doi.org/10.1016/j.cej.2013.11.057

Liu, Z., Dai, Y., Huan, Y., Liu, Z., Sun, L., Zhou, Q., Zhang, W., Sang, Q., Wei, H., Yuan, S., 2013. Different utilizable substrates have different effects on cometabolic fate of imidacloprid in Stenotrophomonas maltophilia. Appl. Microbiol. Biotechnol. 97, 6537–6547. https://doi.org/10.1007/s00253-012-4444-y

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473–474, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065

Ma, F., Guo, J. bo, Zhao, L. jun, Chang, C. chi, Cui, D., 2009. Application of bioaugmentation to improve the activated sludge system into the contact oxidation system treating petrochemical wastewater. Bioresour. Technol. 100, 597–602. https://doi.org/10.1016/j.biortech.2008.06.066

Majewsky, M., Gallé, T., Yargeau, V., Fischer, K., 2011. Active heterotrophic biomass and sludge retention time (SRT) as determining factors for biodegradation kinetics of pharmaceuticals in activated sludge. Bioresour. Technol. 102, 7415–7421. https://doi.org/10.1016/J.BIORTECH.2011.05.032

Major, D.W., McMaster, M.L., Cox, E.E., Edwards, E.A., Dworatzek, S.M., Hendrickson, E.R., Starr, M.G., Payne, J.A., Buonamici, L.W., 2002. Field Demonstration of Successful Bioaugmentation To Achieve Dechlorination of Tetrachloroethene To Ethene. Environ. Sci. Technol. 36, 5106–5116. https://doi.org/10.1021/es0255711

Mamo, J., García-Galán, M.J., Stefani, M., Rodríguez-Mozaz, S., Barceló, D., Monclús, H., Rodriguez-Roda, I., Comas, J., 2018. Fate of pharmaceuticals and their transformation products in integrated membrane systems for wastewater reclamation. Chem. Eng. J. 331, 450–461. https://doi.org/10.1016/J.CEJ.2017.08.050

Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557. https://doi.org/10.1007/s00253-015-7202-0

Martínez, J.L., 2008. Antibiotics and Antibiotic Resistance Genes in Natural Environments. Science (80-.). 321, 365–367. https://doi.org/10.1126/science.1159483

Maurer, M., Escher, B.I., Richle, P., Schaffner, C., Alder, A.C., 2007. Elimination of β-blockers in sewage treatment plants. Water Res. 41, 1614–1622. https://doi.org/10.1016/j.watres.2007.01.004

McAdam, E.J., Bagnall, J.P., Koh, Y.K.K., Chiu, T.Y., Pollard, S., Scrimshaw, M.D., Lester, J.N., Cartmell, E., 2010. Removal of steroid estrogens in carbonaceous and nitrifying activated sludge processes. Chemosphere 81, 1–6. https://doi.org/https://doi.org/10.1016/j.chemosphere.2010.07.057

McClure, N.C., Fry, J.C., Weightman, A.J., 1991. Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated sludge unit. Appl. Environ. Microbiol. 57, 366–373.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995. https://doi.org/http://dx.doi.org/10.1016/j.watres.2012.11.027

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. Environ. Pollut. 157, 1721–1726. https://doi.org/https://doi.org/10.1016/j.envpol.2008.11.045

Min, X., Li, W., Wei, Z., Spinney, R., Dionysiou, D.D., Seo, Y., Tang, C.-J., Li, Q., Xiao, R., 2018. Sorption and biodegradation of pharmaceuticals in aerobic activated sludge system: A combined experimental and theoretical mechanistic study. Chem. Eng. J. 342, 211–219. https://doi.org/10.1016/J.CEJ.2018.01.012

Mrozik, A., Piotrowska-Seget, Z., 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol. Res. 165, 363–375. https://doi.org/10.1016/j.micres.2009.08.001

Müller, E., Schüssler, W., Horn, H., Lemmer, H., 2013. Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as co-substrate and sole carbon and nitrogen source. Chemosphere 92, 969–978. https://doi.org/10.1016/j.chemosphere.2013.02.070

Murdoch, R.W., Hay, A.G., 2005. Formation of Catechols via Removal of Acid Side Chains from Ibuprofen and Related Aromatic Acids. Appl. Environ. Microbiol. 71, 6121–6125. https://doi.org/10.1128/AEM.71.10.6121-6125.2005

Murray, K.E., Thomas, S.M., Bodour, A.A., 2010. Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. Environ. Pollut. 158, 3462–3471. https://doi.org/http://dx.doi.org/10.1016/j.envpol.2010.08.009

Muter, O., Perkons, I., Selga, T., Berzins, A., Gudra, D., Radovica-Spalvina, I., Fridmanis, D., Bartkevics, V., 2017. Removal of pharmaceuticals from municipal wastewaters at laboratory scale by treatment with activated sludge and biostimulation. Sci. Total Environ. 584, 402–413. https://doi.org/10.1016/j.scitotenv.2017.01.023

Nguyen, P.Y., Carvalho, G., Polesel, F., Torresi, E., Rodrigues, A.M., Rodrigues, J.E., Cardoso, V.V., Benoliel, M.J., Plósz, B.G., Reis, M.A.M., Oehmen, A., 2018. Bioaugmentation of activated sludge with Achromobacter denitrificans PR1 for enhancing the biotransformation of sulfamethoxazole and its human conjugates in real wastewater: Kinetic tests and modelling.

Chem. Eng. J. 352, 79–89. https://doi.org/10.1016/J.CEJ.2018.07.011

Nguyen, P.Y., Carvalho, G., Reis, A.C., Nunes, O.C., Reis, M.A.M., Oehmen, A., 2017. Impact of biogenic substrates on sulfamethoxazole biodegradation kinetics by Achromobacter denitrificans strain PR1. Biodegradation 1–13. https://doi.org/10.1007/s10532-017-9789-6

Nunes, B., Carvalho, F., Guilhermino, L., 2005. Acute toxicity of widely used pharmaceuticals in aquatic species: Gambusia holbrooki, Artemia parthenogenetica and Tetraselmis chuii. Ecotoxicol. Environ. Saf. 61, 413–419. https://doi.org/10.1016/j.ecoenv.2004.08.010

Oehmen, A., Marques, R., Noronha, J.P., Carvalho, G., Reis, M.A.M., 2013. Propionate addition enhances the biodegradation of the xenobiotic herbicide propanil and its metabolite. Bioresour. Technol. 127, 195–201. https://doi.org/10.1016/j.biortech.2012.09.120

Park, J., Yamashita, N., Wu, G., Tanaka, H., 2017. Removal of pharmaceuticals and personal care products by ammonia oxidizing bacteria acclimated in a membrane bioreactor: Contributions of cometabolism and endogenous respiration. Sci. Total Environ. 605–606, 18–25. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.06.155

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17, 526–538. https://doi.org/10.1007/s10646-008-0209-x

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. Sci. Total Environ. 371, 314–322. https://doi.org/10.1016/j.scitotenv.2006.07.001

Pereira, V.J., Linden, K.G., Weinberg, H.S., 2007. Evaluation of UV irradiation for photolytic and oxidative degradation of pharmaceutical compounds in water. Water Res. 41, 4413–4423. https://doi.org/10.1016/j.watres.2007.05.056

Perez, S., Eichhorn, P., Aga, D.S., 2005. Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. Environ. Toxicol. Chem. 24, 1361–1367. https://doi.org/10.1897/04-211r.1

Petrie, B., McAdam, E.J., Hassard, F., Stephenson, T., Lester, J.N., Cartmell, E., 2014. Diagnostic investigation of steroid estrogen removal by activated sludge at varying solids retention time. Chemosphere 113, 101–108. https://doi.org/https://doi.org/10.1016/j.chemosphere.2014.04.051

Plósz, B.G., Daigger, G.T., Langford, K.H., Larsen, H.F., Monteith, H., Ort, C., Seth, R., Steyer, J.-P., Vanrolleghem, P.A., 2013a. Modelling micro-pollutant fate in wastewater collection and treatment systems: status and challenges. Water Sci. Technol. 67, 1–15. https://doi.org/10.2166/wst.2012.562

Plósz, B.G., Langford, K.H., Thomas, K. V., 2012. An activated sludge modeling framework for xenobiotic trace chemicals (ASM-X): Assessment of diclofenac and carbamazepine. Biotechnol. Bioeng. 109, 2757–2769. https://doi.org/10.1002/bit.24553

Plósz, B.G., Leknes, H., Lilved, H., Thomas, K. V, 2010a. Diurnal variations in the occurrence and the fate of hormones and antibiotics in activated sludge wastewater treatment in Oslo, Norway. Sci. Tot. Environ. 408, 1915. https://doi.org/10.1016/j.scitotenv.2010.01.042

Plósz, B.G., Leknes, H., Thomas, K. V., 2010b. Impacts of competitive inhibition, parent compound formation and partitioning behavior on the removal of antibiotics in municipal wastewater treatment. Environ. Sci. Technol. 44, 734–742. https://doi.org/10.1021/es902264w

Plósz, B.G., Reid, M.J., Borup, M., Langford, K.H., Thomas, K. V, 2013b. Biotransformation kinetics and sorption of cocaine and its metabolites and the factors influencing their estimation in wastewater. Water Res. 47, 2129–40. https://doi.org/10.1016/j.watres.2012.12.034

Pocostales, J.P., Alvarez, P.M., Beltrán, F.J., 2010. Kinetic modeling of powdered activated carbon

ozonation of sulfamethoxazole in water. Chem. Eng. J. 164, 70–76. https://doi.org/http://dx.doi.org/10.1016/j.cej.2010.08.025

Poi, G., Shahsavari, E., Aburto-Medina, A., Ball, A.S., 2017. Bioaugmentation: an effective commercial technology for the removal of phenols from wastewater. Microbiol. Aust. 38, 82–84. https://doi.org/10.1071/ma17035

Polesel, F., Andersen, H.R., Trapp, S., Plósz, B.G., 2016. Removal of Antibiotics in Biological Wastewater Treatment Systems—A Critical Assessment Using the Activated Sludge Modeling Framework for Xenobiotics (ASM-X). Environ. Sci. Technol. 50, 10316–10334. https://doi.org/10.1021/acs.est.6b01899

Polesel, F., Torresi, E., Loreggian, L., Casas, M.E., Christensson, M., Bester, K., Plósz, B.G., 2017. Removal of pharmaceuticals in pre-denitrifying MBBR – Influence of organic substrate availability in single- and three-stage configurations. Water Res. 123, 408–419. https://doi.org/https://doi.org/10.1016/j.watres.2017.06.068

Pomiès, M., Choubert, J.M., Wisniewski, C., Coquery, M., 2013. Modelling of micropollutant removal in biological wastewater treatments: A review. Sci. Total Environ. 443, 733–748. https://doi.org/http://dx.doi.org/10.1016/j.scitotenv.2012.11.037

Qu, Y., Zhou, J., Wang, J., Song, Z., Xing, L., Fu, X., 2006. Bioaugmentation of Bromoamine Acid Degradation with Sphingomonas xenophaga QYY and DNA Fingerprint Analysis of Augmented Systems. Biodegradation 17, 83–91. https://doi.org/10.1007/s10532-005-3544-0

Qu, Y.Y., Zhou, J.T., Wang, J., Xing, L.L., Jiang, N., Gou, M., Salah Uddin, M., 2009. Population dynamics in bioaugmented membrane bioreactor for treatment of bromoamine acid wastewater. Bioresour. Technol. 100, 244–248. https://doi.org/10.1016/j.biortech.2008.06.023

Quintana, J.B., Weiss, S., Reemtsma, T., 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Res. 39, 2654–2664. https://doi.org/10.1016/j.watres.2005.04.068

Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C., 2000. Stable-isotope probing as a tool in microbial ecology. Nature 403, 646. https://doi.org/10.1038/35001054

Radjenovic, J., Jelic, A., Petrovic, M., Barcelo, D., 2009. Determination of pharmaceuticals in sewage sludge by pressurized liquid extraction (PLE) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Anal. Bioanal. Chem. 393, 1685–1695. https://doi.org/10.1007/s00216-009-2604-4

Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res. 43, 831–841. https://doi.org/http://dx.doi.org/10.1016/j.watres.2008.11.043

Radke, M., Lauwigi, C., Heinkele, G., Múrdter, T.E., Letzel, M., 2009. Fate of the antibiotic sulfamethoxazole and its two major human metabolites in a water sediment test. Environ. Sci. Technol. 43, 3135–3141. https://doi.org/10.1021/es900300u

Rahman, K.S.M., Banat, I.M., Thahira, J., Thayumanavan, T., Lakshmanaperumalsamy, P., 2002. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresour. Technol. 81, 25–32. https://doi.org/https://doi.org/10.1016/S0960-8524(01)00105-5

Ramin, P., Libonati Brock, A., Polesel, F., Causanilles, A., Emke, E., de Voogt, P., Plósz, B.G., 2016. Transformation and Sorption of Illicit Drug Biomarkers in Sewer Systems: Understanding the Role of Suspended Solids in Raw Wastewater. Environ. Sci. Technol. acs.est.6b03049. https://doi.org/10.1021/acs.est.6b03049

Reichert, P., 1994. Aquasim – A tool for simulation and data analysis of aquatic systems. Water Sci. Technol. 30, 21–30.

Reis, P.J.M., Reis, A.C., Ricken, B., Kolvenbach, B.A., Manaia, C.M., Corvini, P.F.X., Nunes, O.C., 2014. Biodegradation of sulfamethoxazole and other sulfonamides by Achromobacter denitrificans PR1. J. Hazard. Mater. 280, 741–749. https://doi.org/http://dx.doi.org/10.1016/j.jhazmat.2014.08.039

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. https://doi.org/https://doi.org/10.1016/j.watres.2007.04.028

Ricken, B., Corvini, P.F.X., Cichocka, D., Parisi, M., Lenz, M., Wyss, D., Martínez-Lavanchy, P.M., Müller, J.A., Shahgaldian, P., Tulli, L.G., Kohler, H.P.E., Kolvenbacha, B.A., 2013. Ipso-hydroxylation and subsequent fragmentation: A novel microbial strategy to eliminate sulfonamide antibiotics. Appl. Environ. Microbiol. 79, 5550–5558. https://doi.org/10.1128/AEM.00911-13

Roeleveld, P.J., Van Loosdrecht, M.C.M., 2002. Experience with guidelines for wastewater characterisation in The Netherlands. Water Sci. Technol. 45, 77–87.

Rogers, H.R., 1996. Sources, behaviour and fate of organic contaminants during sewage treatment and in sewage sludges. Sci. Total Environ. 185, 3–26. https://doi.org/10.1016/0048-9697(96)05039-5

Roh, H., Chu, K.-H., 2011. Effects of solids retention time on the performance of bioreactors bioaugmented with a 17β-estradiol-utilizing bacterium, Sphingomonas strain KC8. Chemosphere 84, 227–233. https://doi.org/http://dx.doi.org/10.1016/j.chemosphere.2011.04.029

Roh, H., Subramanya, N., Zhao, F., Yu, C.-P., Sandt, J., Chu, K.-H., 2009. Biodegradation potential of wastewater micropollutants by ammonia-oxidizing bacteria. Chemosphere 77, 1084–1089. https://doi.org/http://dx.doi.org/10.1016/j.chemosphere.2009.08.049

Rosal, R., Rodríguez, A., Perdigón-Melón, J.A., Petre, A., García-Calvo, E., Gómez, M.J., Agüera, A., Fernández-Alba, A.R., 2010. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Res. 44, 578–588. https://doi.org/10.1016/J.WATRES.2009.07.004

Roth, J.A., 1997. Chapter 14 Principles of drug metabolism, in: Bittar, E.E., Bittar, N. (Eds.), Principles of Medical Biology. Elsevier, pp. 253–280. https://doi.org/https://doi.org/10.1016/S1569-2582(97)80076-1

Sacher, F., Lange, F.T., Brauch, H.J., Blankenhorn, I., 2001. Pharmaceuticals in groundwaters: Analytical methods and results of a monitoring program in Baden-Württemberg, Germany. J. Chromatogr. A 938, 199–210. https://doi.org/10.1016/S0021-9673(01)01266-3

Sahar, E., Messalem, R., Cikurel, H., Aharoni, A., Brenner, A., Godehardt, M., Jekel, M., Ernst, M., 2011. Fate of antibiotics in activated sludge followed by ultrafiltration (CAS-UF) and in a membrane bioreactor (MBR). Water Res. 45, 4827–4836. https://doi.org/10.1016/j.watres.2011.06.023

Santharam, S., Ibbini, J., Davis, L.C., Erickson, L.E., 2011. Field study of biostimulation and bioaugmentation for remediation of tetrachloroethene in groundwater. Remediat. J. 21, 51–68. https://doi.org/10.1002/rem.20281

Sathyamoorthy, S., Chandran, K., Ramsburg, C.A., 2013. Biodegradation and cometabolic modeling of selected beta blockers during ammonia oxidation. Environ. Sci. Technol. 47, 12835–12843. https://doi.org/10.1021/es402878e

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science 313, 1072–1077.

Seshan, H., Goyal, M.K., Falk, M.W., Wuertz, S., 2014. Support vector regression model of wastewater bioreactor performance using microbial community diversity indices: Effect of stress and bioaugmentation. Water Res. 53, 282–296. https://doi.org/10.1016/j.watres.2014.01.015

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 Res. 38, 2323–2330. https://doi.org/http://dx.doi.org/10.1016/j.watres.2004.02.022

Sonnenschein, C., Soto, A.M., 1998. An updated review of environmental estrogenandandrogen mimicsandantagonists. J. Steroid Biochem. Mol. Biol. 65, 143–150.

Spark, K.M., Swift, R.S., 2002. Effect of soil composition and dissolved organic matter on pesticide sorption. Sci. Total Environ. 298, 147–161. https://doi.org/https://doi.org/10.1016/S0048-9697(02)00213-9

Stadler, L.B., Ernstoff, A.S., Aga, D.S., Love, N.G., 2012. Micropollutant Fate in Wastewater Treatment: Redefining "Removal." Environ. Sci. Technol. 46, 10485–10486. https://doi.org/10.1021/es303478w

Stadler, L.B., Su, L., Moline, C.J., Ernstoff, A.S., Aga, D.S., Love, N.G., 2015. Effect of redox conditions on pharmaceutical loss during biological wastewater treatment using sequencing batch reactors. J. Hazard. Mater. 282, 106–115. https://doi.org/10.1016/j.jhazmat.2014.08.002

Stasinakis, A.S., Kordoutis, C.I., Tsiouma, V.C., Gatidou, G., Thomaidis, N.S., 2010. Removal of selected endocrine disrupters in activated sludge systems: Effect of sludge retention time on their sorption and biodegradation. Bioresour. Technol. 101, 2090–2095. https://doi.org/https://doi.org/10.1016/j.biortech.2009.10.086

Su, L., Aga, D., Chandran, K., Khunjar, W.O., 2015. Factors impacting biotransformation kinetics of trace organic compounds in lab-scale activated sludge systems performing nitrification and denitrification. J. Hazard. Mater. 282, 116–124. https://doi.org/10.1016/j.jhazmat.2014.08.007

Suárez, S., Lema, J.M., Omil, F., 2010. Removal of Pharmaceutical and Personal Care Products (PPCPs) under nitrifying and denitrifying conditions. Water Res. 44, 3214–3224. https://doi.org/http://dx.doi.org/10.1016/j.watres.2010.02.040

Suárez, S., Ramil, M., Omil, F., Lema, J.M., 2005. Removal of pharmaceutically active compounds in nitrifying–denitrifying plants. Water Sci. Technol. 52, 9–14.

Suárez, S., Reif, R., Lema, J.M., Omil, F., 2012. Mass balance of pharmaceutical and personal care products in a pilot-scale single-sludge system: Influence of T, SRT and recirculation ratio. Chemosphere 89, 164–171. https://doi.org/10.1016/j.chemosphere.2012.05.094

Tadkaew, N., Hai, F.I., McDonald, J.A., Khan, S.J., Nghiem, L.D., 2011. Removal of trace organics by MBR treatment: The role of molecular properties. Water Res. 45, 2439–2451. https://doi.org/https://doi.org/10.1016/j.watres.2011.01.023

Tadkaew, N., Sivakumar, M., Khan, S.J., McDonald, J.A., Nghiem, L.D., 2010. Effect of mixed liquor pH on the removal of trace organic contaminants in a membrane bioreactor. Bioresour. Technol. 101, 1494–1500. https://doi.org/10.1016/j.biortech.2009.09.082

Taheran, M., Brar, S.K., Verma, M., Surampalli, R.Y., Zhang, T.C., Valero, J.R., 2016. Membrane processes for removal of pharmaceutically active compounds (PhACs) from water and wastewaters. Sci. Total Environ. 547, 60–77. https://doi.org/https://doi.org/10.1016/j.scitotenv.2015.12.139

Tamtam, F., Mercier, F., Eurin, J., Chevreuil, M., Le Bot, B., 2009. Ultra performance liquid chromatography tandem mass spectrometry performance evaluation for analysis of antibiotics in natural waters. Anal. Bioanal. Chem. 393, 1709–1718. https://doi.org/10.1007/s00216-008-2576-9

Tan, D.T., Arnold, W.A., Novak, P.J., 2013. Impact of organic carbon on the biodegradation of estrone in mixed culture systems. Environ. Sci. Technol. 47, 12359–65. https://doi.org/10.1021/es4027908

Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904. https://doi.org/10.1016/j.chemosphere.2006.06.035

Ternes, T.A., Herrmann, N., Bonerz, M., Knacker, T., Siegrist, H., Joss, A., 2004. A rapid method to measure the solid-water distribution coefficient (K d) for pharmaceuticals and musk fragrances in sewage sludge. Water Res. 38, 4075–4084. https://doi.org/10.1016/j.watres.2004.07.015

Ternes, T.A., Joss, A., 2006. Human Pharmaceuticals, Hormones and Fragrances, in: IWA Publishing , London, UK. pp. 19–24.

Ternes, T.A., Kreckel, P., Mueller, J., 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants — II. Aerobic batch experiments with activated sludge. Sci. Total Environ. 225, 91–99. https://doi.org/https://doi.org/10.1016/S0048-9697(98)00335-0

Testa, B., Pedretti, A., Vistoli, G., 2012. Reactions and enzymes in the metabolism of drugs and other xenobiotics. Drug Discov. Today 17, 549–560. https://doi.org/10.1016/j.drudis.2012.01.017

Thompson, I.P., Van Der Gast, C.J., Ciric, L., Singer, A.C., 2005. Bioaugmentation for bioremediation: The challenge of strain selection. Environ. Microbiol. 7, 909–915. https://doi.org/10.1111/j.1462-2920.2005.00804.x

Tian, S., Qian, C., Yang, X., 2006. Biodegradation of biomass gasification wastewater by two species of Pseudomonas using immobilized cell reactor. Appl. Biochem. Biotechnol. 128, 141–147. https://doi.org/10.1385/ABAB:128:2:141

Tolls, J., 2001. Sorption of veterinary pharmaceuticals in soils: A review. Environ. Sci. Technol. 35, 3397–3406. https://doi.org/10.1021/es0003021

Torresi, E., Escolà Casas, M., Polesel, F., Plósz, B.G., Christensson, M., Bester, K., 2017. Impact of external carbon dose on the removal of micropollutants using methanol and ethanol in post-denitrifying Moving Bed Biofilm Reactors. Water Res. 108, 95–105. https://doi.org/https://doi.org/10.1016/j.watres.2016.10.068

Torresi, E., Fowler, S.J., Polesel, F., Bester, K., Andersen, H.R., Smets, B.F., Pl??sz, B.G., Christensson, M., 2016. Biofilm thickness influences biodiversity in nitrifying MBBRs - Implications on micropollutant removal. Environ. Sci. Technol. 50, 9279–9288. https://doi.org/10.1021/acs.est.6b02007

Tran, N.H., Urase, T., Kusakabe, O., 2009. The characteristics of enriched nitrifier culture in the degradation of selected pharmaceutically active compounds. J. Hazard. Mater. 171, 1051–1057. https://doi.org/http://dx.doi.org/10.1016/j.jhazmat.2009.06.114

Tran, N.H., Urase, T., Ngo, H.H., Hu, J., Ong, S.L., 2013. Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants. Bioresour. Technol. 146, 721–731. https://doi.org/10.1016/J.BIORTECH.2013.07.083

Trovó, A.G., Nogueira, R.F.P., Agüera, A., Sirtori, C., Fernández-Alba, A.R., 2009. Photodegradation of sulfamethoxazole in various aqueous media: Persistence, toxicity and photoproducts assessment. Chemosphere 77, 1292–1298. https://doi.org/10.1016/j.chemosphere.2009.09.065

Tyagi, M., da Fonseca, M.M.R., de Carvalho, C., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22, 231–241. https://doi.org/10.1007/s10532-010-9394-4

van't Hoff, M.J.H., 1884. Etudes de dynamique chimique. Recl. des Trav. Chim. des Pays-Bas 3, 333–336. https://doi.org/10.1002/recl.18840031003

Van der Ven, A., Mantel, M., Vree, T., Koopmans, P., Van der Meer, J., 1994. Formation and elimination of sulphamethoxazole hydroxylamine after oral administration of sulphamethoxazole. Br. J. Clin. Pharmacol. 38, 147–150. https://doi.org/10.1111/j.1365-2125.1994.tb04339.x

van der Ven, A.J., Vree, T.B., van Ewijk-Beneken Kolmer, E.W., Koopmans, P.P., van der Meer, J.W., 1995. Urinary recovery and kinetics of sulphamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulphamethoxazole. Br. J. Clin. Pharmacol. 39, 621–625.

van der Zee, F.P., Villaverde, S., 2005. Combined anaerobic–aerobic treatment of azo dyes—A short review of bioreactor studies. Water Res. 39, 1425–1440. https://doi.org/https://doi.org/10.1016/j.watres.2005.03.007

Van Limbergen, H., Top, E.M., Verstraete, W., 1998. Bioaugmentation in activated sludge: current features and future perspectives. Appl. Microbiol. Biotechnol. 50, 16–23. https://doi.org/https://doi.org/10.1007/s002530051250

Verlicchi, P., Al Aukidy, M., Zambello, E., 2015. What have we learned from worldwide experiences on the management and treatment of hospital effluent? — An overview and a discussion on perspectives. Sci. Total Environ. 514, 467–491. https://doi.org/https://doi.org/10.1016/j.scitotenv.2015.02.020

Wagner, M., Loy, A., Nogueira, R., Purkhold, U., Lee, N., Daims, H., 2002. Microbial community composition and function in wastewater treatment plants. Antonie Van Leeuwenhoek 81, 665–680. https://doi.org/10.1023/A:1020586312170

Wang, J., Gardinali, P.R., 2014. Identification of phase II pharmaceutical metabolites in reclaimed water using high resolution benchtop Orbitrap mass spectrometry. Chemosphere 107, 65–73. https://doi.org/10.1016/j.chemosphere.2014.03.021

Wang, J., Liu, G.-F., Lu, H., Jin, R.-F., Zhou, J.-T., Lei, T.-M., 2012. Biodegradation of Acid Orange 7 and its auto-oxidative decolorization product in membrane-aerated biofilm reactor. Int. Biodeterior. Biodegradation 67, 73–77. https://doi.org/http://dx.doi.org/10.1016/j.ibiod.2011.12.003

Wang, J., Wang, S., 2018. Microbial degradation of sulfamethoxazole in the environment. Appl. Microbiol. Biotechnol. 102, 3573–3582. https://doi.org/10.1007/s00253-018-8845-4

Wang, L., Chi, X.-Q., Zhang, J.-J., Sun, D.-L., Zhou, N.-Y., 2014. Bioaugmentation of a methyl parathion contaminated soil with Pseudomonas sp. strain WBC-3. Int. Biodeterior. Biodegradation 87, 116–121. https://doi.org/http://dx.doi.org/10.1016/j.ibiod.2013.11.008

Wang, L., Liu, Y., Ma, J., Zhao, F., 2016. Rapid degradation of sulphamethoxazole and the further transformation of 3-amino-5-methylisoxazole in a microbial fuel cell. Water Res. 88, 322–328. https://doi.org/10.1016/j.watres.2015.10.030

Watkinson, A.J., Murby, E.J., Costanzo, S.D., 2007. Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. Water Res. 41, 4164–4176. https://doi.org/10.1016/j.watres.2007.04.005

Watkinson, A.J., Murby, E.J., Kolpin, D.W., Costanzo, S.D., 2009. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. Sci. Total Environ. 407, 2711–2723. https://doi.org/http://dx.doi.org/10.1016/j.scitotenv.2008.11.059

Wen, D., Zhang, J., Xiong, R., Liu, R., Chen, L., 2013. Bioaugmentation with a pyridine-degrading bacterium in a membrane bioreactor treating pharmaceutical wastewater. J. Environ. Sci. 25,

2265–2271. https://doi.org/http://dx.doi.org/10.1016/S1001-0742(12)60278-2

Wu, H., Shen, J., Jiang, X., Liu, X., Sun, X., Li, J., Han, W., Wang, L., 2018. Bioaugmentation strategy for the treatment of fungicide wastewater by two triazole-degrading strains. Chem. Eng. J. 349, 17–24. https://doi.org/10.1016/J.CEJ.2018.05.066

Xu, N., Johnson, A.C., Jürgens, M.D., Llewellyn, N.R., Hankins, N.P., Darton, R.C., 2009. Estrogen concentration affects its biodegradation rate in activated sludge. Environ. Toxicol. Chem. 28, 2263–70. https://doi.org/10.1897/08-577.1

Xu, W., Zhang, G., Zou, S., Li, X., Liu, Y., 2007. Determination of selected antibiotics in the Victoria Harbour and the Pearl River, South China using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Environ. Pollut. 145, 672–679. https://doi.org/10.1016/j.envpol.2006.05.038

Yang, S., Cha, J., Carlson, K., 2005. Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry. J. Chromatogr. A 1097, 40–53. https://doi.org/10.1016/j.chroma.2005.08.027

Yao, Y., Lu, Z., Zhu, F., Min, H., Bian, C., 2013. Successful bioaugmentation of an activated sludge reactor with Rhodococcus sp. YYL for efficient tetrahydrofuran degradation. J. Hazard. Mater. 261, 550–558. https://doi.org/http://dx.doi.org/10.1016/j.jhazmat.2013.08.007

Yi, T., Harper, W.F., 2007. The Link between Nitrification and Biotransformation of 17α-Ethinylestradiol. Environ. Sci. Technol. 41, 4311–4316. https://doi.org/10.1021/es070102q

Yu, T.-H., Lin, A.Y.-C., Lateef, S.K., Lin, C.-F., Yang, P.-Y., 2009. Removal of antibiotics and non-steroidal anti-inflammatory drugs by extended sludge age biological process. Chemosphere 77, 175–181. https://doi.org/http://dx.doi.org/10.1016/j.chemosphere.2009.07.049

Yu, Z., Mohn, W.W., 2002. Bioaugmentation with the resin acid-degrading bacterium Zoogloea resiniphila DhA-35 to counteract pH stress in an aerated lagoon treating pulp and paper mill effluent. Water Res. 36, 2793–2801. https://doi.org/http://dx.doi.org/10.1016/S0043-1354(01)00496-1

Zhang, D., Gersberg, R.M., Ng, W.J., Tan, S.K., 2014. Removal of pharmaceuticals and personal care products in aquatic plant-based systems: A review. Environ. Pollut. 184, 620–639. https://doi.org/https://doi.org/10.1016/j.envpol.2013.09.009

Zhang, Y.-B., Zhou, J., Xu, Q.-M., Cheng, J.-S., Luo, Y.-L., Yuan, Y.-J., 2016. Exogenous cofactors for the improvement of bioremoval and biotransformation of sulfamethoxazole by Alcaligenes faecalis. Sci. Total Environ. 565, 547–556. https://doi.org/https://doi.org/10.1016/j.scitotenv.2016.05.063

Zhou, N.A., Lutovsky, A.C., Andaker, G.L., Gough, H.L., Ferguson, J.F., 2013. Cultivation and characterization of bacterial isolates capable of degrading pharmaceutical and personal care products for improved removal in activated sludge wastewater treatment. Biodegradation 24, 813–827. https://doi.org/10.1007/s10532-013-9630-9

Ziels, R.M., Lust, M.J., Gough, H.L., Strand, S.E., Stensel, H.D., 2014. Influence of Bioselector Processes on 17α-Ethinylestradiol Biodegradation in Activated Sludge Wastewater Treatment Systems. Environ. Sci. Technol. 48, 6160–6167. https://doi.org/10.1021/es405351b

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. Lancet 355, 1789–1790. https://doi.org/10.1016/S0140-6736(00)02270-4

APPENDIX A

Appendix A1. Direct HPLC injection of sulfamethoxazole, sulfanilic acid and 3-amino-5-methylisoxazole

For the tests of 150 mg L^{-1} and 500 µg L^{-1}, samples were taken for pH determination, and then centrifuged at 10000 rpm for 5 minutes in a VWR Microstar 17 centrifuge to remove the biomass, filtered through a 0.2 µm Spin Filter (Centrifugal filter modified nylon, VWR), and stored in 1.5 mL Eppendorf tubes at -20ºC for the monitoring of SMX concentration and degradation metabolites. All selected organic compounds were analysed by HPLC using a Waters system equipped with ultraviolet (UV) and fluorescence detectors (Waters Chromatography, Milford, MA, USA) and a Luna 5µm C18(2) 100A (150 x 3.0 mm) column (Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of water acidified with formic acid 0.1% v/v (A): acetonitrile (B) at a flow rate of 1.2 mL/min and the eluting conditions applied consisted of 1.5 min at 5% of B; 3 min of a linear gradient up to 100% of B and finally 2.5 min at 5% B. The volume of injection was 50 µL. Samples containing the mixtures of compounds were analysed using the conditions described in Table A1.

Table A1. HPLC methods used for the detection of SMX and biodegradation metabolites in the direct HPLC analysis

Compound	Mobile Phase Composition (%)	Column temperature (°C)	Flow rate (mL/min)	HPLC/UV $Wavelength_{UV}$ (nm)
Sulfamethoxazole	5%ACN/95%H_2O acidified with formic acid 0.1% (v/v)	35	1.2	270
Sulfanilic acid	5%ACN/95%H_2O acidified with formic acid 0.1% (v/v)	35	1.2	230
3-amino-5-methylisoxazole	5%ACN/95%H_2O acidified with formic acid 0.1% (v/v)	35	1.2	250

ACN - Acetonitrile; MeOH - Methanol; H_2O - Milli-Q water; UV - Ultraviolet;

Appendix A2. Direct LC-MS/MS injection of sulfamethoxazole and 3-amino-5-methylisoxazole

For the tests of 20 µg L^{-1} and 600 ng L^{-1}, the analyses were performed using a high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. This equipment is equipped with a binary pump, an automatic injector and a thermostatted column compartment coupled to a Mass Spectrometer TSQ Endura triple quadrupole model, from Thermo Scientific. The separation was performed on a reversed-phase column (Acquity BEH C18 (2,1 x 50 mm, 1,7 µm), Waters)) at 40ºC using an injection volume of 20 µL. The mobile phase consisted of water:formic acid 0.5% v/v supplemented with 0.01 mM ammonium

acetate (A): methanol (B) at a flow rate of 0.30 mL/min and the eluting conditions applied consisted of 2 min at 5% of B; 2 min at 20% of B, 2 min at 40% of B followed by 2 more minutes at 70% of B then a linear gradient up to 90% of B for 2 min before finally reduced to 5 % of B for the last 4 min.

Triple quadrupole operating conditions were optimized in order to work in multiple reaction monitoring mode (MRM). The optimization was based on the selection of ionization mode, optimum collision energy (eV), ion transfer tube. MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in Table A2. The first transition corresponds to the most abundant and was used for quantification and the second one for confirmation purposes.

XCalibur software (version 4.1) was used for data acquisition and processing.

Ultrahigh-purity Argon (Ar) was used as collision gas. High purity nitrogen was used as sheath, aux and sweep gas.

Table A2. MS/MS parameters for the analysis of target analytes by MRM negative and positive ionization mode

Target compounds	**R_t (min)**	**Precursor ion $[M+H]^+$**	**Collision energy (eV)**	**MRM1**	**Collision Energy**	**MRM2**
Sulfamethoxazole		254.1	15	254.1>156	20	254.1>92
D4-sulfamethoxazole		258.2	15	258.2>160.1	25	258.2>96.1
3-amino-5-methylisoxazole		99.1	10	99>72	12	99>44

Table A3. Structure and physico-chemical properties of the sulfamethoxazole

Compound	**Molecular formula**	**Molecular Weight (Da)**	**Solubility**	**Log K_{ow}[a]**	**CAS**	**K_{oc}**	Vapor pressure (mmHg)	Log K_d	pK_a
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.3	610 mg L^{-1} (37^{0}C)[1]	0.89[2]	723-46-6	4.44[3] (pH 7	1.3E-7[3]	2.4[4]	1.9/5.7

[a] Log K_{ow}: logarithm of the octanol-water partition coefficient

[1](Yang et al. 2011); [2](Kolpin 2002); [3](Park and Choi 2008); [4](Gobel et al. 2005)

APPENDIX B

Appendix B1. Metabolism of sulfamethoxazole in the human body

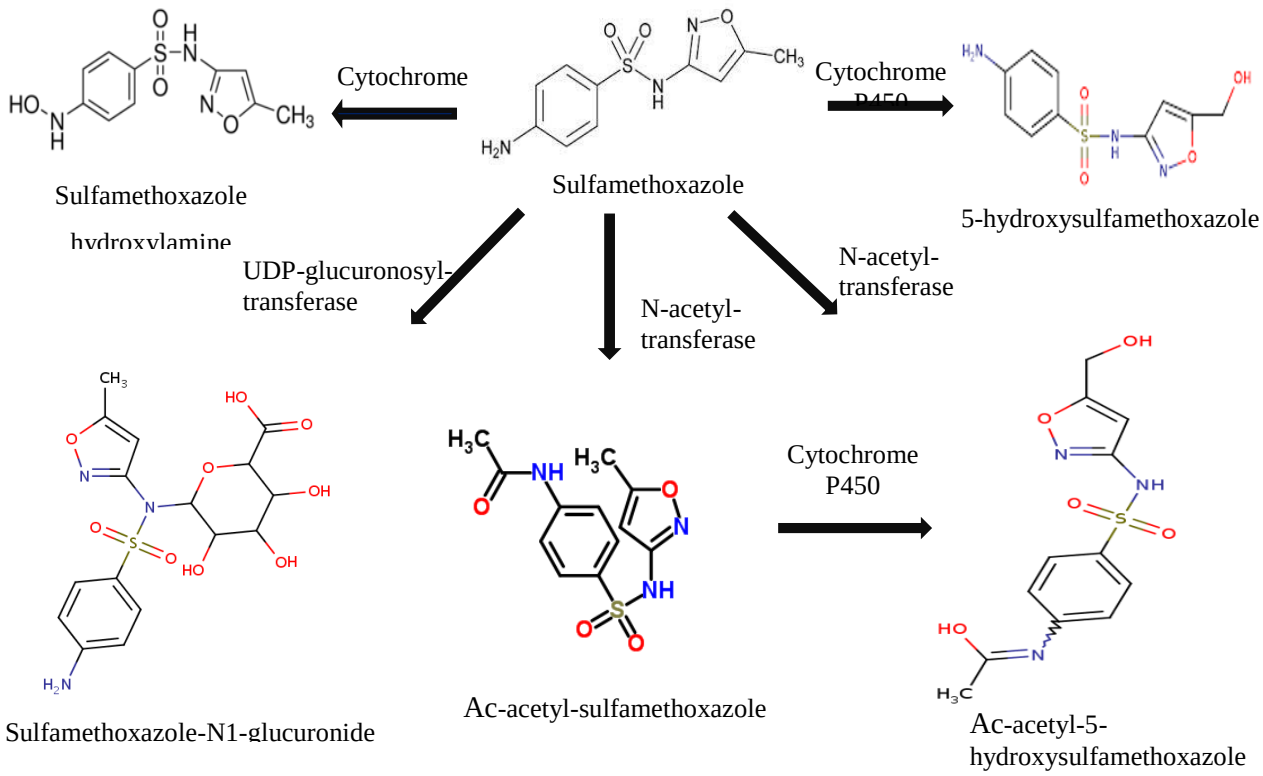


Figure B1. Metabolism of sulfamethoxazole in humans, adapted from Radke et al. (2009)

Amongst five conjugated metabolites of SMX (Figure B1), only two metabolites (i.e. Ac-SMX and SMX-Glu) were detected in the environment (Bonvin et al. 2011; Göbel et al. 2007; Hilton and Thomas 2003; Stadler et al. 2015) and represented high amount of total load of SMX, i.e. 52-58% of the source of sulfamethoxazole in reclaimed water (Wang and Gardinali 2014), suggesting the importance of measuring these two human metabolites.

Appendix B2. Sample preparation and analytical methods for the concentration of sulfamethoxazole, the two human metabolites and biodegradation metabolites with LC-MS/MS

Sample preparation

The following SPE procedure was based on a previously published method for the analysis of sulfonamides in natural waters (Ye et al. 2007). The sample was filtered through nylon syringe filter 0.2 µm (Whatman). The supernatant was stored in 10 mL glass vials until analysis (within 2 weeks). Before performing SPE, the sample aliquot was added with Na_2EDTA solution as a complexing agent, and was spiked with surrogate standards d4-N_4-acetyl-sulfamethoxazole, sulfamethoxazole-d4-N_1-glucuronide, sulfamethoxazole-d4 each at 500 ng L^{-1}, adjusted to pH = 3. Isotope labelled compounds were used to correct for any losses that may have occurred during SPE and quantify the compounds

while accounting for matrix effects inherent to wastewater samples. Analytes were extracted using the hydrophilic-lipophilic balance OASIS HLB catridge (6 mL, 200 mg) from Waters (Millford, MA). The cartridge was pre-conditioned with 6 mL of MeOH, followed by 3 mL of acidified methanol (0.1% formic acid in HPLC grade methanol, v/v), and then 2 x 6 mL of MilliQ-water. After that, samples were extracted through the HLB cartridges at a flow rate of ~5 mL/min using a 20-position vacuum manifold (Waters). After extraction, the cartridge was rinsed with 2 x 6 mL of MilliQ-water and vacuum-dried for ~5 min. The retained analytes were subsequently eluted with 4 x 2 mL of acidified methanol (50 mM formic acid) into a glass test tube. The SPE eluent was evaporated to dryness under a gentle flow of nitrogen and finally reconstituted to 500 µL in a solvent mixture of MilliQ-water:methanol (9:1). The extract was transferred to an amber autosampler vial, and stored at -20ºC until LC-MS/MS analysis, which was carried out the day after.

Analytical methods

The concentration of SMX and their metabolites were monitored by using a high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. This equipment is equipped with a binary pump, an automatic injector and a thermostatted column compartment coupled to a Mass Spectrometer TSQ Endura triple quadrupole model, from Thermo Scientific. The separation was performed on a reversed-phase column (Acquity BEH C18 (2,1 x 50 mm, 1,7 µm), Waters)) at 40ºC using an injection volume of 20 µL. The mobile phase consisted of water:formic acid 0.5% v/v supplemented with 0.01 mM ammonium acetate (A): methanol (B) at a flow rate of 0.30 mL/min and the eluting conditions applied consisted of 2 min at 5% of B; 2 min at 20% of B, 2 min at 50% of B followed by 2 more minutes at 70% of B then a linear gradient up to 90% of B for 2 min before finally reduced to 5 % of B for the last 3 min.

Triple quadrupole operating conditions were optimized in order to work in multiple reaction monitoring mode (MRM). The optimization was based on the selection of ionization mode, optimum collision energy (eV).

Ionization was achieved by positive electron spray ionization (ESI), using a spray voltage of 4 kV situated at a 90°angle to the entrance. Drying gas temperature was set as 350°C, nebulizer pressure (N_2) as 22 psi and drying gas flow rate as 11 L/min to achieve the highest sensitivity. Ultrahigh-purity Argon (Ar) was used as collision gas. High purity nitrogen was used as sheath, auxiliary and sweep gas.

MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in Table B1. The first transition corresponds to the most abundant and was used for quantification and the second one for confirmation purposes.

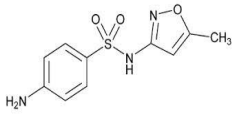
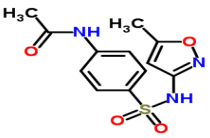
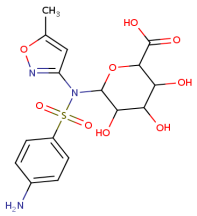
XCalibur software (version 4.1) was used for data acquisition and processing.

Table B1. MS/MS parameters for the analysis of target analytes by MRM positive ionization mode

Target compounds	t_R (min)	Precursor ion $[M+H]^+$	MRM1		MRM2	
			Collision energy (eV)	Production ion	Collision Energy (eV)	Production ion
Sulfamethoxazole	5.84	254.1	15	156	20	92
N_4-acetyl-sulfamethoxazole	6.81	296.3	25	134.1	18	198.1
Sulfamethoxazole-N_1-glucuronide	4.93	430.3	10	254.3	30	156.1
d4-sulfamethoxazole	5.80	258.2	15	160.1	25	96.1
d4-N_4-acetyl-sulfamethoxazole	6.82	300.3	25	138.2	18	202.2
Sulfamethoxazole-d4-N_1-glucuronide	4.87	434.3	12	258.3	30	160.1
3-amino-5-methylisoxazole	0.89	99.1	10	99.2>72	12	99>44

Target compounds	t_R (min)	Precursor ion $[M+H]^+$	Collision energy (eV)	MRM1	Collision Energy (eV)	MRM2
Sulfamethoxazole	5.84	254.1	15	156	20	92
N_4-acetyl-sulfamethoxazole	6.81	296.3	25	134.1	18	198.1
sulfamethoxazole-N_1-glucuronide	4.93	430.3	10	254.3	30	156.1
d4-sulfamethoxazole	5.80	258.2	15	160.1	25	96.1
d4-N_4-acetyl-sulfamethoxazole	6.82	300.3	25	138.2	18	202.2
sulfamethoxazole-d4-N_1-glucuronide	4.87	434.3	12	258.3	30	160.1
3-amino-5-methylisoxazole	0.89	99.1	10	99.2>72	12	99>44

Table B2. Target pharmaceuticals and transformation products under investigation: structure, properties, and wastewater concentration (K_{ow} – octanol–water partition coefficient; K_d – solid–liquid partition coefficient; pK_a–acid dissociation constant; TP – transformation product; N.F – not found).

Chemical	Structure	Use	Log K_{ow}	Log K_d	pK_a	WWTP primary effluent incidence concentration (µg L^{-1})
Sulfamethoxazole		Antibiotic	0.89[a]	2.4[b]	pK_{a1} = 1.8[c] pK_{a2} = 5.7[c]	0.87 ± 0.75
N_4-acetyl-sulfamethoxazole		Antibiotic TP	N.F	N.F	5.6 ± 0.5[c]	0.98 ± 0.2
Sulfamethoxazole-N_1-glucuronide		Antibiotic TP	1.21	N.F	2.7 ± 0.5[c]	n.d.

n.d. : not determined; [a](Kolpin et al., 2002); [b](Göbel et al., 2005); [c](Radke et al., 2009)

Appendix B3. Chelas Wastewater Treatment Plant (Lisbon, Portugal)

Municpal WWTP Chelas was designed to receive about 52500 m^3 of wastewater per day, with a capacity of 211000 population equivalents (PE). The WWTP comprises various treatment processes such as a pre-treatment, primary treatment, biological treatment (anoxic-aerobic process), tertiary treatment (sand filtration → UV) and sludge treatment. The biological treatment was designed for nitrogen removal with a pre-denitrificcation process and operated at hydraulic retention time (HRT) of 2 hours. Biogas produced from the anaerobic digestion process of sludge treatment is used as energy to lower the plant operational cost. Characteristics of the primary effluent wastewater are mentioned in Table B3. The average treatment performance of Chelas WWTP was 90% removal of $N\text{-}NH4^+$, 60% removal of $N\text{-}NO_3^-$.

Table B3. Primary effluent wastewater characteristics

	TCOD (mg L^{-1}) (n=23)	sCOD (mg L^{-1}) (n=23)	BOD_5 (mg L^{-1}) (n=13)	$N\text{-}NH_4^+$ (mg L^{-1}) (n=76)	SMX (µg L^{-1}) (n=3)	Ac-SMX (µg L^{-1}) (n=3)
Range	126-500	50-139	33-143	40-53	0.52 – 1.73	0.78 -1.17
Mean ± std	258 ± 97	99 ± 22	91 ± 37	40 ± 8	0.87 ± 0.75	0.98 ± 0.20

TCOD: total COD; sCOD: soluble COD

Table B4. Goodness of the fit (R2) of the models used in this study

Test	Compound	Biotransformation		Retransformation
		Pseudo-first order kinetic	Cometabolic enhancement kinetic	Pseudo-first order kinetic
A1	SMX	0.04	0.95	
	Ac-SMX			0.99
	SMX-Glu			0.98
A2	SMX	0.52	0.79	
	Ac-SMX			0.99
	SMX-Glu			0.98
A3	SMX	0.94	0.95	
	Ac-SMX			0.99
	SMX-Glu			0.98
A4	SMX	0.89	0.99	
	Ac-SMX			0.98
	SMX-Glu			0.94
An1	SMX	0.99		
	Ac-SMX			0.99
	SMX-Glu			0.99
An2	SMX	0.98		
	Ac-SMX			0.99
	SMX-Glu			0.99

Table B5. Parameters of the ASM model (Henze et al. 2000) used and calibrated in this study

Parameter	Definition	Values	Unit
μ_H	Specific growth rate of heterotrophs	Calibrated	day^{-1}
Y_H	Yield coefficient for heterotrophs	0.67	g cell COD formed (g COD oxidized)$^{-1}$
K_S	Saturation constant for substrate S_S	20	gCOD m^{-3}

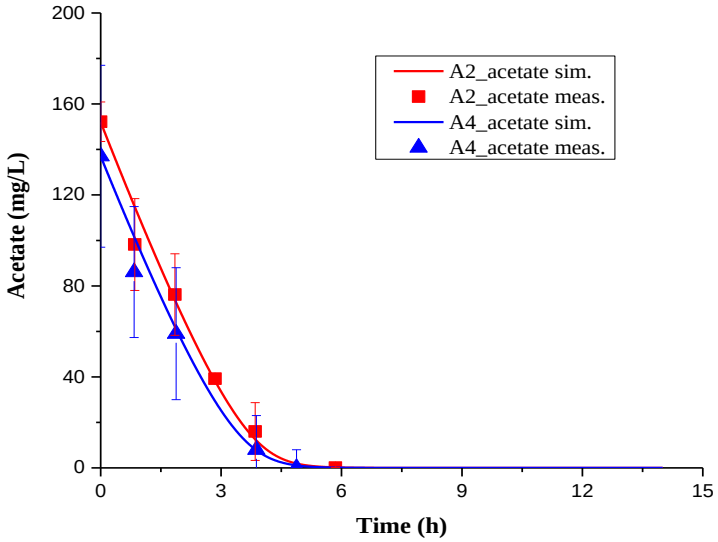


Figure B2. Measured and simulated acetate, expressed as sCOD (mg L^{-1})for aerobic batch tests (A1): non-bioaugmented AS with supplementation of acetate; and (A4): bioaugmented AS with supplementation of acetate. Error bars indicates standard deviation for duplicates

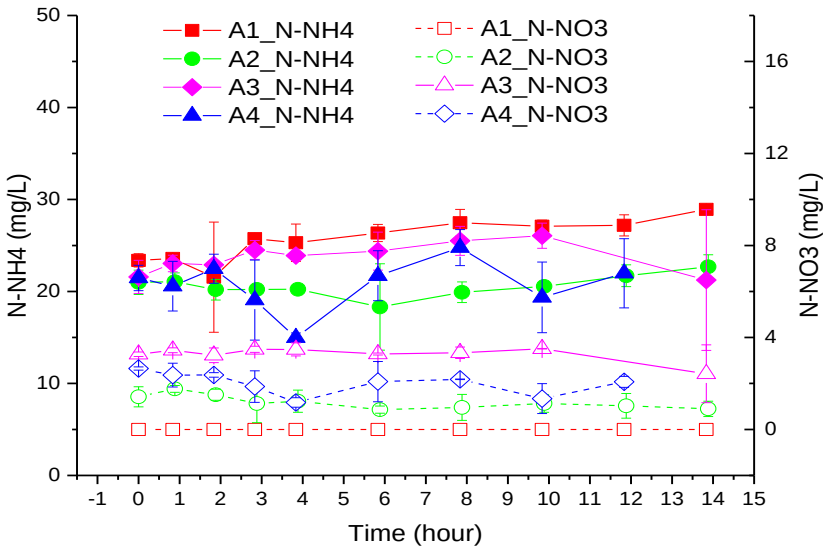


Figure B3. Evolution of ammonium concentration for aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplementation of acetate as additional C-source; (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* supplemented with acetate test. Error bars indicate standard deviations for duplicates.

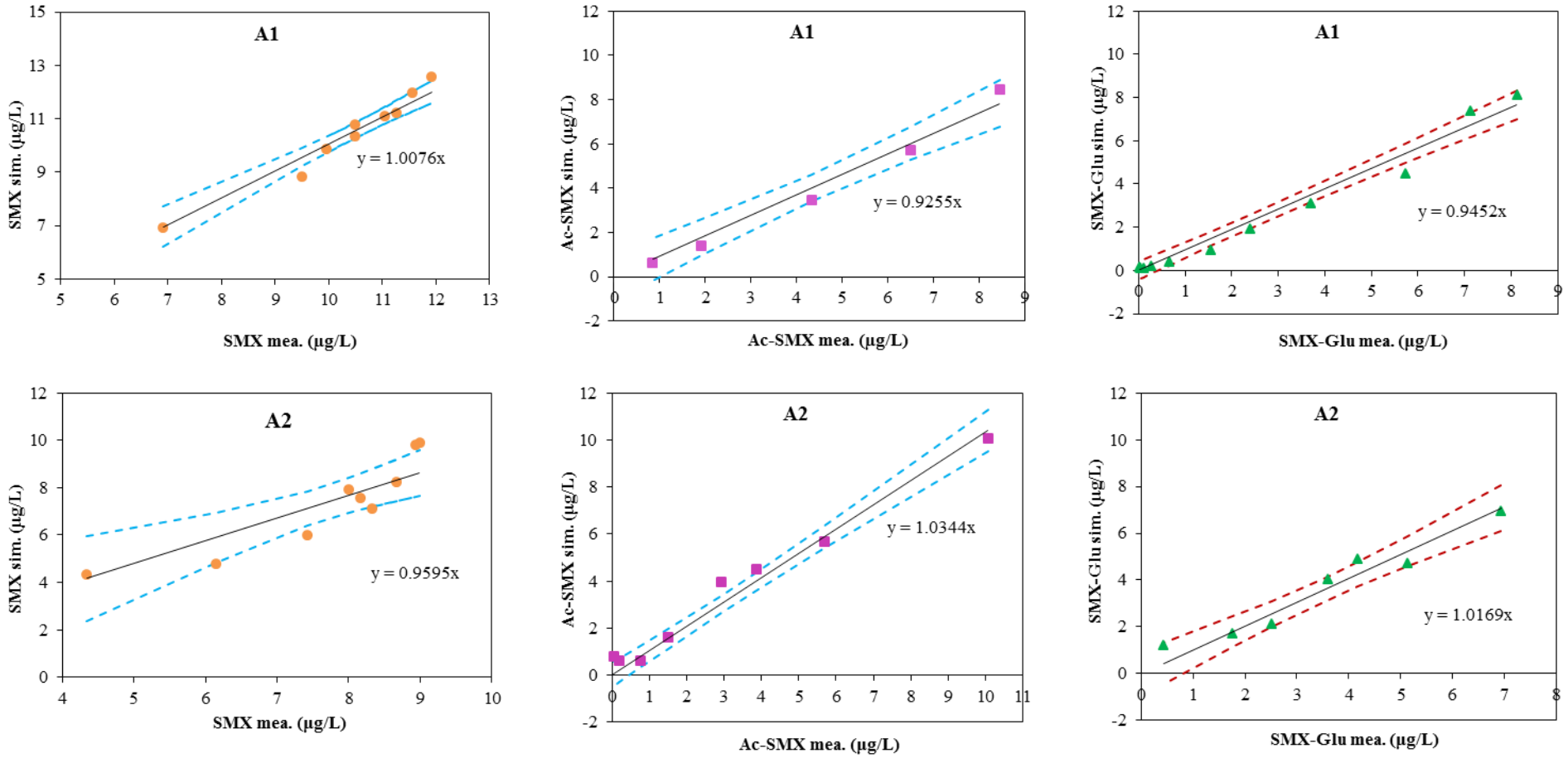


Figure B4. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the non-bioaugmented aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplement of acetate as additional C-source. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.

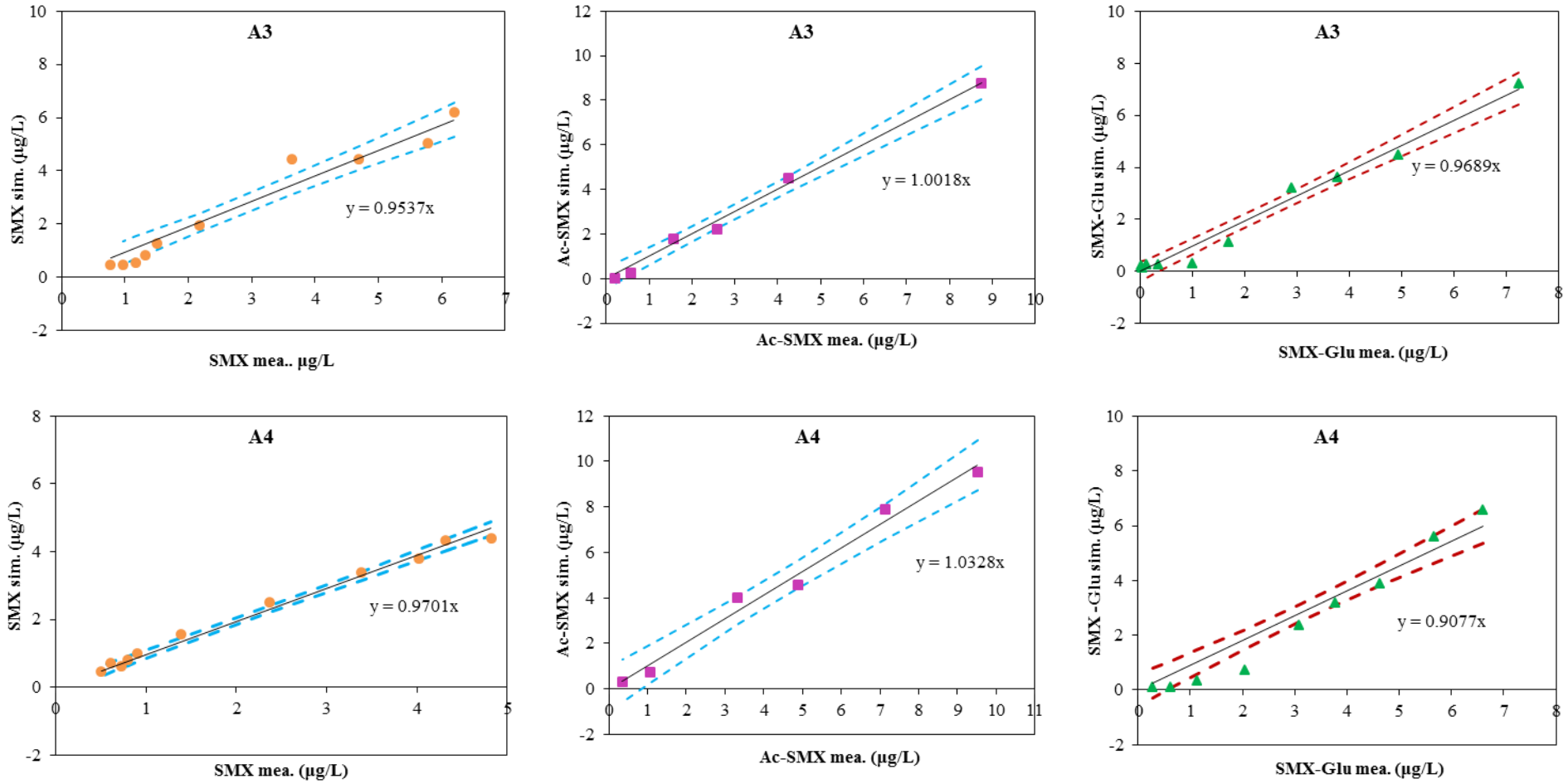


Figure B5. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the bioaugmented aerobic batch tests (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* PR1 supplement with acetate test. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.

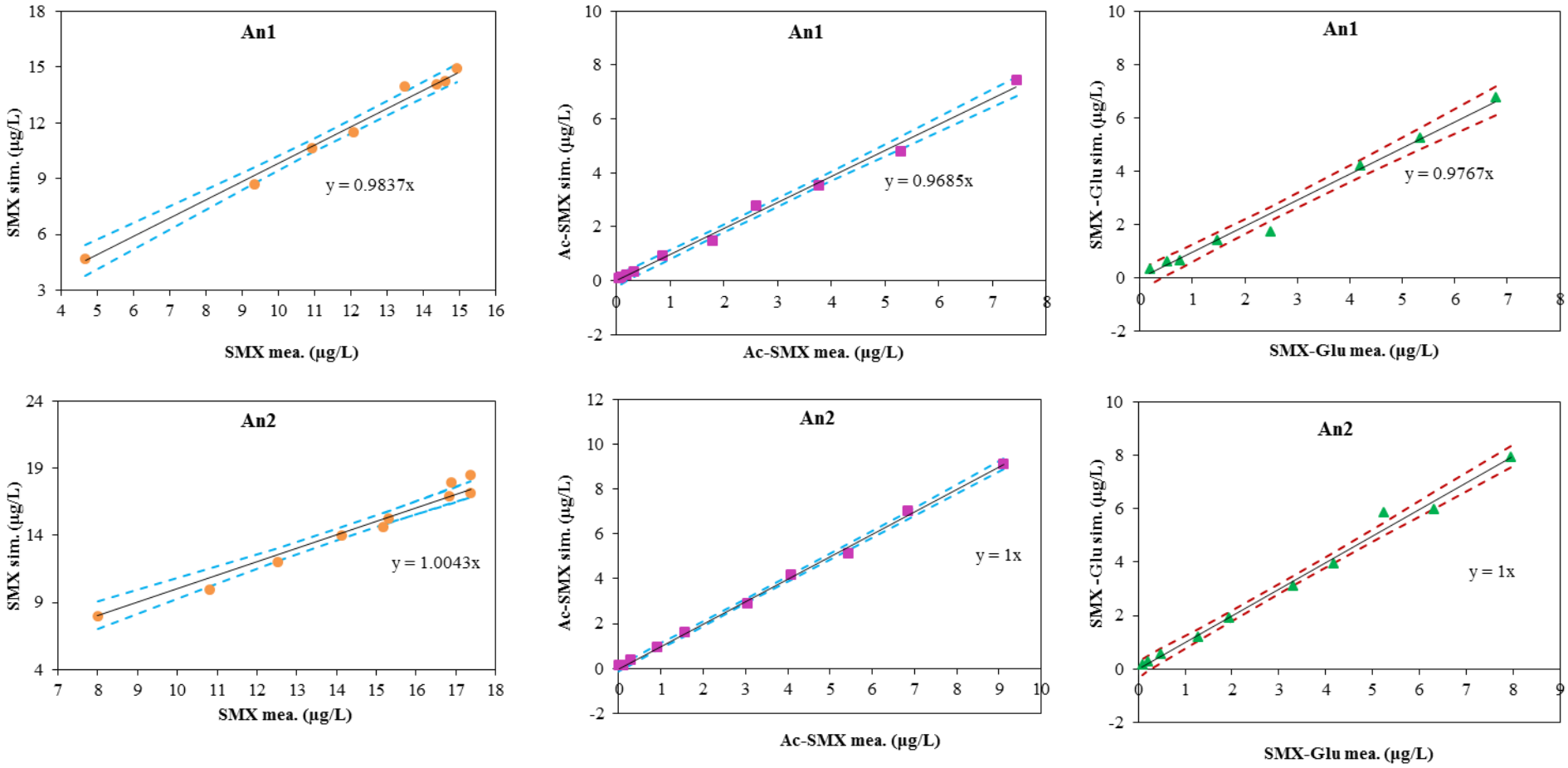


Figure B6. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the anoxic batch tests (An1): non-bioaugmented activated sludge; (An2): bioaugmented activated sludge with *A. deninitrificans* PR1. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.

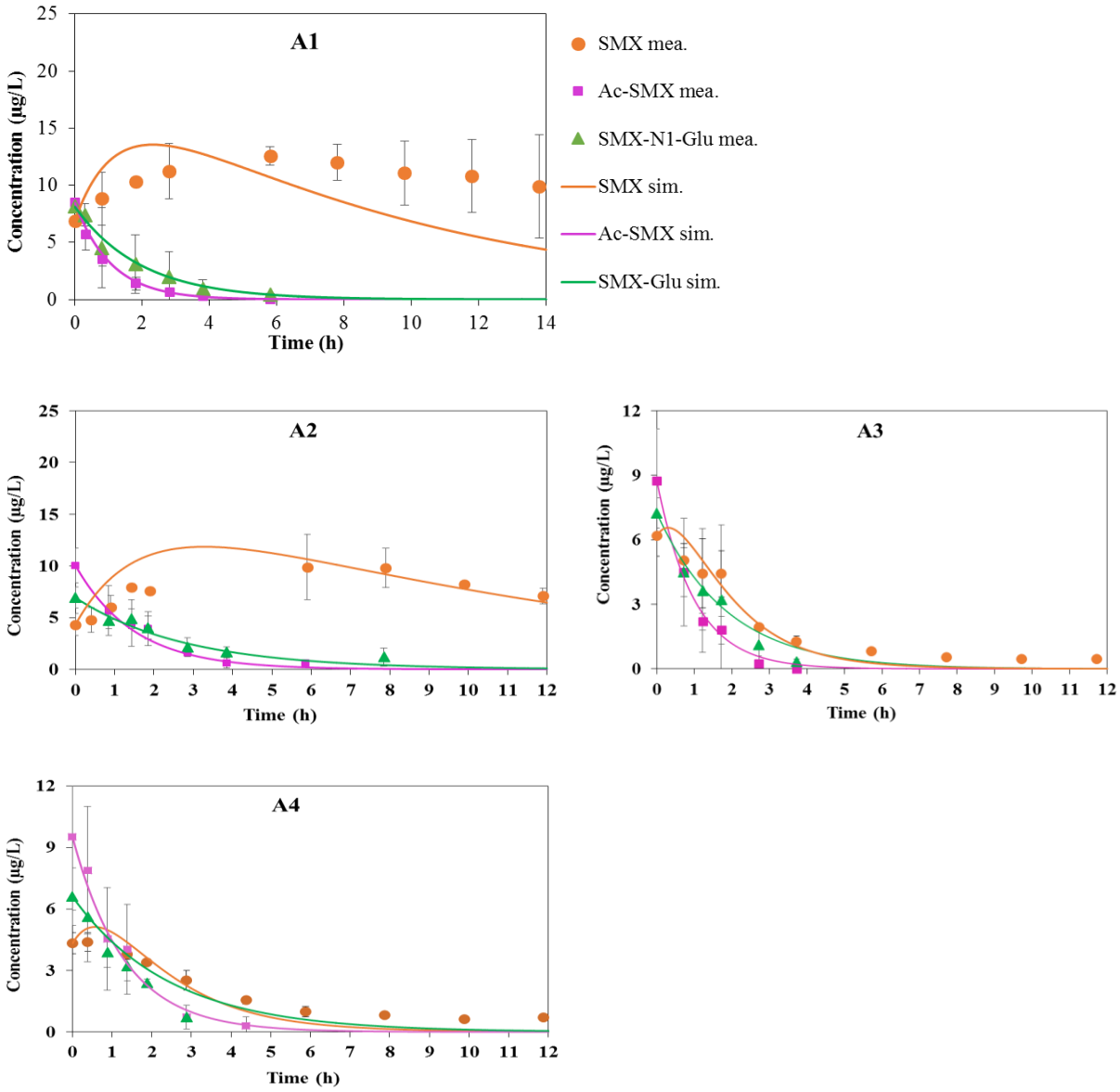


Figure B7. Illustration of measured concentrations of SMX, Ac-SMX, and SMX-Glu (markers) and simulated (lines) as a function of time for aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplementation of acetate as additional C-source; (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* PR1 supplemented with acetate test. Error bars indicate the standard deviations for duplicates

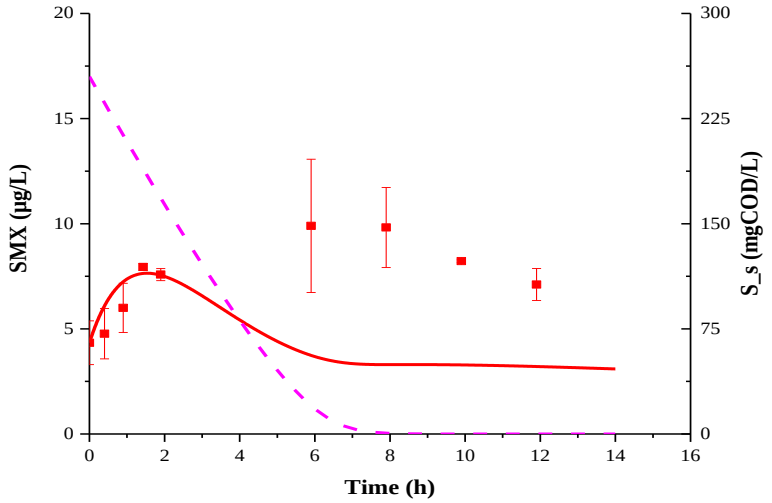


Figure B8. Illustration of measured (makers) and simulated (continuous lines) concentrations of SMX as a function of time for aerobic batch tests (A2): non-bioaugmented AS test with supplementation of acetate as additional C-source, with both acetate and other readily biodegradable substrates that present in wastewater (expressed as sCOD) were considered as the primary substrates (S_S) in the cometabolic model to enhance the SMX biotransformation by AS

APPENDIX C

Appendix C1. Sample preparation and analytical methods

Analytical methods

The concentration of SMX and their metabolites were monitored by using a high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a Dionex Ultimate 3000 system from Thermo Scientific. This equipment is equipped with a binary pump, an automatic injector and a thermostatted column compartment coupled to a Mass Spectrometer TSQ Endura triple quadrupole model, from Thermo Scientific. The separation was performed on a reversed-phase column (Acquity BEH C18 (2,1 x 50 mm, 1,7 µm), Waters)) at 40ºC using an injection volume of 20 µL. The mobile phase consisted of water:formic acid 0.5% v/v supplemented with 0.01 mM ammonium acetate (A): methanol (B) at a flow rate of 0.30 mL/min and the eluting conditions applied consisted of 2 min at 5% of B; 2 min at 20% of B, 2 min at 50% of B followed by 2 more minutes at 70% of B then a linear gradient up to 90% of B for 2 min before finally reduced to 5 % of B for the last 3 min.

Triple quadrupole operating conditions were optimized in order to work in multiple reaction monitoring mode (MRM). The optimization was based on the selection of ionization mode, optimum collision energy (eV).

Ionization was achieved by positive electron spray ionization (ESI), using a spray voltage of 4 kV situated at a 90˚angle to the entrance. Drying gas temperature was set as 350˚C, nebulizer pressure (N_2) as 22 psi and drying gas flow rate as 11 L/min to achieve the highest sensitivity. Ultrahigh-purity Argon (Ar) was used as collision gas. High purity nitrogen was used as sheath, auxiliary and sweep gas.

MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in Table C1. The first transition corresponds to the most abundant and was used for quantification and the second one for confirmation purposes.

XCalibur software (version 4.1) was used for data acquisition and processing.

Table C1. MS/MS parameters for the analysis of target analytes by MRM negative and positive ionization mode

Target compounds	t_R (min)	Precursor ion $[M+H]^+$	MRM1		MRM2	
			Collision energy (eV)	Production ion	Collision Energy (eV)	Production ion
Sulfamethoxazole	5.84	254.1	15	156	20	92
N_4-acetyl-sulfamethoxazole	6.81	296.3	25	134.1	18	198.1
Sulfamethoxazole-N_1-glucuronide	4.93	430.3	10	254.3	30	156.1
d4-sulfamethoxazole	5.80	258.2	15	160.1	25	96.1
d4-N_4-acetyl-sulfamethoxazole	6.82	300.3	25	138.2	18	202.2
Sulfamethoxazole-d4-N_1-glucuronide	4.87	434.3	12	258.3	30	160.1
3-amino-5-methylisoxazole	0.89	99.1	10	99.2>72	12	99>44

Appendix C2. Chelas Wastewater Treatment Plant (Lisbon, Portugal)

Chelas was designed to receive about 52500 m^3 of wastewater per day, with a capacity of 211000 population equivalents (PE). The WWTP comprises various treatment processes such as a pre-treatment, primary treatment, biological treatment (anoxic-aerobic process), tertiary treatment (sand filtration → UV) and sludge treatment. The biological treatment was designed for nitrogen removal with a pre-denitrificcation process and operated at hydraulic retention time (HRT) of 2 hours. Biogas produced from the anaerobic digestion process of sludge treatment is used as energy to lower the plant operational cost. The average treatment performance of Chelas WWTP was approx. 90% removal of $N\text{-}NH4^+$ and 60% removal of $N\text{-}NO_3^-$.