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# **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**

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1 **Abstract**

2 *Achromobacter denitrificans* PR1 has previously shown potential to degrade the antibiotic sulfamethoxazole,  
3 whereby sulfamethoxazole biotransformation was stimulated in the presence of biogenic substrates. This study  
4 examined the biotransformation kinetics of sulfamethoxazole and its two main conjugates, N<sub>4</sub>-acetyl-SMX  
5 and SMX-N<sub>1</sub>-Glucuronide, by activated sludge and activated sludge bioaugmented with *A. denitrificans* PR1.  
6 SMX biotransformation under both anoxic and aerobic conditions was tested, with and without the addition of  
7 acetate as growth substrate, to understand the range of applicable conditions for bioaugmentation purposes.  
8 Biological process models, such as the pseudo-first order kinetic and cometabolic models, were also applied  
9 and, following the estimation of kinetic parameters, could well describe data measured in bioaugmented and  
10 non-bioaugmented AS batch experiments under various test conditions. Experimental and modelling results  
11 suggest that (i) retransformation of the two conjugates to SMX in AS occurred under both aerobic and anoxic  
12 conditions, and (ii) biotransformation kinetics of SMX can vary significantly depending on redox conditions,  
13 e.g., SMX was biotransformed by AS only under aerobic conditions. Notably, SMX biotransformation was  
14 significantly enhanced when PR1 was bioaugmented in AS. Addition of acetate as biogenic substrate is not  
15 necessary, as PR1 was capable of enhancing the SMX biotransformation by using the carbon sources present  
16 in wastewater. Overall, bioaugmentation by means of *A. denitrificans* PR1 could be a viable strategy for  
17 enhancing SMX removal in AS wastewater treatment plants (WWTPs).

18

19 **Keywords:** antibiotics, cometabolism, N<sub>4</sub>-acetyl-SMX, SMX-N<sub>1</sub>-Glucuronide, modelling, retransformation

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## 27 1. Introduction

28 The intensive use of antibiotics for human and veterinary therapy has led to their continuous discharge, also  
29 in the form of conjugates, in the environment. WWTPs are not designed to remove these and other xenobiotic  
30 chemicals, and discharge of treated effluents into the environment has been a major concern due to the risk of  
31 a worldwide dispersal of antibiotic resistance genes [1].

32 Amongst antibiotics, sulfamethoxazole (SMX) is one of the most widely used synthetic sulfonamides  
33 worldwide. SMX enters WWTPs via human excretion in the forms of unchanged SMX (15-25% of the  
34 excreted dose) as well as the conjugated forms N<sub>4</sub>-acetyl-SMX (Ac-SMX) (> 40%) and SMX-N<sub>1</sub>-Glucuronide  
35 (SMX-Glu) (9-15%) [2]. The two human conjugates have been detected in wastewater influent and effluent,  
36 and were observed to rapidly deconjugate during wastewater treatment [3,4] which was considered to likely  
37 explain the reported 'negative removal' of SMX in wastewater treatment [3,5]. This suggests the importance  
38 of investigating the retransformation of the two major human conjugates to parent SMX, in order to explain  
39 the reported differences in removal efficiencies in WWTPs [6,7].

40 SMX removal has been shown to vary greatly, i.e. from negative (-138%) to very high (>90%) [1] in full-scale  
41 WWTPs, and with variability in SMX biotransformation kinetics. SMX was also shown to be not readily  
42 biodegradable during the 28-day test period in a closed bottle test [8].

43 Biotransformation has been recognized as the major elimination mechanism of SMX and its conjugates during  
44 biological treatment of domestic wastewater, with minor contribution of sorption onto sludge (due to the polar  
45 nature of these compounds). Overall, literature reports of inconsistent and incomplete SMX elimination  
46 suggest that novel technologies/strategies would be required if more stringent discharge limits for SMX and  
47 other antibiotics are enforced. Bioaugmentation can be an alternative WWTP operational strategy to enable or  
48 enhance xenobiotics removal by inoculating specialized degrading bacteria [9]. Despite the fact that  
49 bioaugmentation has been studied for years in wastewater treatment to reinforce biological processes, few  
50 studies have tested the use of bioaugmentation for enhancing the removal of xenobiotics, e.g. 17 $\beta$ -estradiol  
51 [10], estradiol [11], fungicides [12]. With respect to antibiotics, bioaugmentation resulted in limited SMX  
52 removal when applying *Microbacterium* sp. strain BR1 in full-scale membrane bioreactors [13], except for  
53 SMX concentrations far higher than the ones normally found in municipal wastewater.

54 Previously, we showed that a pure culture of *Achromobacter denitrificans* PR1 could exhibit faster  
55 biotransformation kinetics (up to two to three orders of magnitude higher) of SMX compared to AS alone [14],  
56 even at the low SMX concentrations typical of wastewater effluents. Given its ability to degrade SMX in the  
57 presence and/or absence of other additional carbon sources (acetate and succinate) at environmentally relevant  
58 concentrations (typical of e.g., wastewater effluents), the strain likely has potential for treating SMX in  
59 wastewater upon bioaugmentation. Therefore, the overall objective of this work was to investigate whether  
60 PR1 can enhance SMX biotransformation kinetics when bioaugmented to AS with real wastewater feed.  
61 Specifically, we (i) investigated the effect of redox conditions, i.e. aerobic and anoxic conditions, on the  
62 transformation rates of targeted compounds; (ii) assessed the potential influence of retransformation processes  
63 of the two main human conjugates, i.e. Ac-SMX and SMX-Glu, on the fate of sulfamethoxazole under the  
64 testing conditions; and (iii) evaluated the need for supplementation with a biogenic substrate (e.g. acetate) or  
65 whether the availability of carbon sources in wastewater could serve as biogenic substrates to achieve a  
66 sufficiently interesting kinetic for SMX removal upon bioaugmentation of AS with PR1. Modelling the fate of  
67 xenobiotics in WWTPs can be a useful tool to understand their removal mechanisms, predict and reduce their  
68 emissions with treated effluent through process optimization. Specifically, the Activated Sludge Modelling  
69 framework for Xenobiotics (ASM-X), has been previously used to predict the fate of SMX in biological  
70 treatment systems [7] and to identify factors (influent concentration of conjugates, solid residence time)  
71 possibly explaining the variability in SMX removal efficiencies [15]. In this context, suitable mathematical  
72 models were developed to examine the metabolic mechanism and predict kinetics of SMX and human  
73 conjugates biotransformation upon bioaugmentation of *A. denitrificans* PR1 into AS.

## 74 **2. Materials and methods**

### 75 **2.1. Chemicals and reagents**

76 Reagent grade (purity  $\geq 99\%$ ) SMX was purchased from Sigma-Aldrich. Ac-SMX, SMX-Glu and isotopically  
77 labelled Ac-SMX-d4, SMX-d4-Glu, SMX-d4 were obtained from Toronto Research Chemicals, Inc. (TRC,  
78 Canada). Individual stock standard solutions were prepared on a weight basis in methanol and stored at  $-20^{\circ}\text{C}$ .  
79 HPLC-grade methanol was supplied by Merck (Darmstadt, Germany).

## 80 2.2. Laboratory-scale experiments - Bioaugmentation of AS with *A. denitrificans* PR1

### 81 2.2.1. Culture media

82 Bacterial inoculum was grown in mineral medium B, supplemented with ammonium phosphate at  
83 concentration of 400 mM (designated as MMBN), as previously described by [16]. The cells were harvested  
84 by centrifugation (7000 x g for 10 min at 20°C using a Sigma® 4-16KS centrifuge), and rinsed three times  
85 with fresh MMBN medium to remove the trace amount of SMX remaining from the culture medium before  
86 augmenting to the reactors to get an initial cell suspension concentration of approximately 0.05-0.06 mg<sub>biomass</sub>  
87 L<sup>-1</sup>.

### 88 2.2.2. Batch tests

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

95 For all experiments, primary effluent wastewater and AS (from a Modified Ludzack Ettinger system) collected  
96 from the Chelas WWTP (Lisbon, Portugal) were used. More information about Chelas WWTP is provided in  
97 Section S2 and Table S3, Supplementary Information (SI). AS and primary effluent were seeded to the 1 L  
98 glass reactors at an initial biomass concentration of approximately 3 gTSS L<sup>-1</sup> for all the experiments. Overall,  
99 four types of batch tests were performed: (i) abiotic control tests; (ii) sorption tests; (iii) bioaugmentation tests;  
100 (iv) nitrification inhibition tests. The testing conditions are presented in Table 1. All the tests were performed  
101 in duplicate, except for the anoxic bioaugmented AS test (An2, Table 1), the control 1 and the allylthiourea  
102 (ATU) nitrification inhibition tests.

#### 103 *Abiotic control test (control 1)*

104 The goal of this experiment was to determine the contribution of abiotic removal mechanisms (stripping,  
105 sorption onto reactor walls and equipment, and abiotic chemical reactions). In this test, the 1 L-glass-reactor

106 was filled with Milli-Q water that was spiked with the three compounds, e.g. SMX, Ac-SMX and SMX-Glu  
107 at the concentrations of  $10 \mu\text{g L}^{-1}$ ,  $15 \mu\text{g L}^{-1}$  and  $15 \mu\text{g L}^{-1}$ , respectively. The experiment lasted 6 hours.

#### 108 *Sorption tests (control 2)*

109 Sodium azide ( $\text{NaN}_3$ ) is a well know respiration inhibitor used for negative control tests in AS studies. A wide  
110 range of concentrations from  $0.5$  to  $720 \text{ mg}_{\text{azide}} \text{g}_{\text{TSS}}^{-1}$  were used in previous studies for this purpose [17]. In this  
111 test, a concentration of  $\sim 650 \text{ mg}_{\text{azide}} \text{g}_{\text{TSS}}^{-1}$  was used to inhibit AS activity. SMX, Ac-SMX and SMX-Glu were  
112 spiked into the reactors at the initial concentrations of  $5 \mu\text{g.L}^{-1}$ ,  $10 \mu\text{g.L}^{-1}$  and  $10 \mu\text{g.L}^{-1}$ , respectively. The tests  
113 were performed in duplicate.

#### 114 *Bioaugmentation tests*

115 The goal of these tests was to assess biotransformation of the targeted compounds with non-bioaugmented and  
116 bioaugmented AS with *A. denitrificans* PR1. Batch experiments were performed during 12 to 14 hours under  
117 aerobic and anoxic conditions. In aerobic tests, the influence of a biogenic substrate on SMX biotransformation  
118 was assessed by adding acetate at an initial concentration of  $\sim 137$  to  $152 \text{ mg}_{\text{COD}} \text{L}^{-1}$  that is similar to the level  
119 of soluble COD typically found in many activated sludge WWTPs.

120 In anoxic batch tests, reactors were supplemented with an initial nitrate concentration of  $80 \text{ mg NO}_3\text{-N L}^{-1}$  in  
121 the form of  $\text{KNO}_3$ . Aqueous stock solutions of SMX and the two target conjugates were spiked to obtain an  
122 initial concentration of approximately  $5 \mu\text{g L}^{-1}$  and  $10 \mu\text{g L}^{-1}$ , respectively.

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130 **Table 1. Overview of the different tested conditions in the bioaugmented and non-bioaugmented AS**  
 131 **experiments**

		Batch	Feed	AS (gTSS L <sup>-1</sup> )	<i>A.denitrificans</i> PR1 (g <sub>biomass</sub> L <sup>-1</sup> )	Acetate (mg <sub>COD</sub> L <sup>-1</sup> )	NaN <sub>3</sub> (mg <sub>azide</sub> g <sub>TSS</sub> <sup>-1</sup> )	ATU (mg L <sup>-1</sup> )
Control	Control 1	C1	MilliQ- water	-	-	-		
	Control 2	C2	WW*	~ 3	-	-	~ 650	
Nitrification- inhibition		ATU	WW*	~ 3	-	-		30
		Without ATU	WW*	~ 3	-	-	-	-
Bioaugment ation 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		

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

134 *Nitrification inhibition tests*

135 To determine the contribution of ammonia oxidizing bacteria and heterotrophs to the SMX biotransformation  
 136 in AS communities, biomass was inactivated using ATU at 30 mg L<sup>-1</sup> [18], a copper chelator that depletes



137 copper ions from the active center of ammonia monooxygenases (AMO), therefore inhibiting ammonia  
138 oxidizing activity.

### 139 **2.3. Sample preparation and analytical procedures**

140 Samples collected along the tests were centrifuged for 5 min at 8000 xg, followed by syringe filtration through  
141 0.2 µm cellulose Whatman filters and stored at -20°C prior to analysis of soluble chemicals.

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

145 Total and volatile suspended solids (TSS, VSS) were determined according to Standard Methods (APHA,  
146 1995). Ammonium, nitrate and nitrite concentrations were measured using a segmented flow analyzer through  
147 the Skalar San++ system. Samples were also analyzed for soluble COD (sCOD) using HACH-lange test kits  
148 and a DR2800 spectrophotometer (HACH, Germany).

149 Analysis of SMX and the two human conjugates was performed on a high performance liquid chromatography  
150 coupled to tandem mass spectrometry (HPLC-MS/MS) using a Dionex Ultimate 3000 system from Thermo  
151 Scientific. Detailed descriptions of the sample preparation and analytical methods used are provided as Section  
152 S1 (SI).

### 153 **2.4. Determination of kinetic parameters-modelling approach**

#### 154 **2.4.1. Modelling assumptions**

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

#### 160 **2.4.2. Model implementation and estimation of parameters**

161 In this study, the biotransformation rate of the three target compounds was calibrated using the ASM-X  
162 modelling framework [7,15,19].

163 Deconjugation of the two human conjugates (Ac-SMX and SMX-Glu) to form the parent compound SMX is  
164 described by a pseudo-first order kinetic model (Table 2, process (1) for aerobic and process (9) for anoxic  
165 removal), thus allowing the estimation of the biotransformation rate coefficients, e.g.  $k_{Dec,Ox}$  or  $k_{Dec,Ax}$  ( $L\ gTSS^{-1}\ d^{-1}$ ).  
166  $d^{-1}$ ).

167 For the biotransformation of SMX under aerobic conditions, both pseudo-first order and cometabolic models  
168 were implemented to test which one could appropriately predict SMX biotransformation (Table 2). The  
169 cometabolic biotransformation model [19] consisted of two biotransformation rates: the enhanced rate in the  
170 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.  
171 Accordingly, biotransformation kinetics of the cometabolic substrate (e.g. micropollutants) depend on the  
172 readily biodegradable growth substrates,  $S_S$  ( $mgCOD\ L^{-1}$ ).  $S_S$  was determined as the difference between soluble  
173 COD (sCOD, measured during the experiments) and soluble inert COD ( $S_I$  – calculated according to [20]).  
174 The initial  $S_S$  concentration of the pre-clarified municipal wastewater used in this study ranged between 41  
175 and  $128\ mgCOD\ L^{-1}$ . Parameters that could not be identified through model calibration to experimental results  
176 (i.e. heterotrophic yields  $Y_H$ , substrate affinity constant  $K_S$ ) were adopted from literature [21]. Concentration  
177 profiles of acetate, expressed as sCOD, were used to calibrate the maximum specific growth rate of  
178 heterotrophs  $\mu_H$  (Table S5 and Fig. S1, SI). The estimated parameters included: (i) biotransformation rate  
179 constants of the AS ( $k_{bio,AS}$ ) and the bioaugmented strain ( $k_{bio,PR1}$ ) in the absence of primary substrate; and (ii)  
180 the cometabolic biotransformation rate constants of the AS ( $q_{bio,AS}$ ) and the bioaugmented strain ( $q_{bio,PR1}$ ) in  
181 the presence of the primary substrates. Each batch test was designed to determine a specific kinetic constant  
182 and is described in Table 3. The model was implemented in Aquasim 2.1d [22] and the embedded secant  
183 method was used for parameter estimation.

184 In our study, experimental data from A1, A4, An1 and An2 tests were used for the model calibration and  
185 estimation of the biotransformation rate constants of SMX and the two human conjugates by AS and *A.*  
186 *denitrificans* PR1 under aerobic and anoxic conditions (Table 3). More details on the model calibration  
187 procedure are presented in Supplementary Information (section S3).

188 2.4.3. *Model validation*

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

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192 **Table 2. Stoichiometric (Gujer) matrix of the ASM-X for processes of parent compound retransformation, biotransformation and the alternative**  
 193 **cometabolic biotransformation model. Parameters and state variables for determination of micropollutants kinetics are described in the main text.**

Processes i → j process ↓	$C_{LI}$	$C_{CJ}$	$S_s$	$X_{AS}$	$X_{PRI}^*$	Process rate
<b>Pseudo-first order kinetics – Aerobic processes</b>						
(1) Parent compound formation due to retransformation of human	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. <i>A. denitrificans</i> <i>PRI</i>	-1				*	$\frac{k_{bio\_PRI} C_{LI} X_{PRI}}{1 + K_D X_{PRI}}$
<b>Cometabolic model – Aerobic processes</b>						
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 bioaugmentation strain, i.e. <i>A. denitrificans</i> strain <i>PRI</i>					*	$\frac{(q_{bio\_PRI} \frac{S_s}{S_s + K_S} + k_{bio\_PRI}) C_{LI} X_{PRI}}{1 + K_D X_{AS}}$
(6) Aerobic growth				*	*	$\mu_H \frac{S_s}{S_s + K_S} X_H$

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**Pseudo first order kinetics – Anoxic processes**

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

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

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

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

197  $C_{LI}$  and  $C_{CJ}$ : the aqueous concentrations of the parent compound and the human conjugates undergoing deconjugation to the parent compound, respectively ( $\mu\text{g L}^{-1}$ ).

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

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

200  $k_{bio\_PR1}$ : is the reaction rate coefficient of biotransformation of parent compound ( $\text{L gTSS}^{-1} \text{d}^{-1}$ ) by the bioaugmented *A. denitrificans* strain PR1.

201  $K_S$ : half-saturation coefficient for  $S_s$

202  $K_D$ : sorption coefficient ( $0.256 \text{ L gbiomass}^{-1}$  for SMX - [23]). The values are not available for  $N_4$ -acetyl-SMX and SMX- $N_1$ -Glucuronide, and were therefore assumed to be equal to 0.

203  $X_{PR1}$  or  $X_{AS}$  : biomass concentration of bio-augmented strain *A. denitrificans* or AS, expressed in gTSS L<sup>-1</sup>;

204  $X_H$  is expressed in gCOD L<sup>-1</sup> by assuming biomass-to-COD ratio of 0.75

205 **Table 3. Model calibration and parameter estimation procedures for the batch tests performed under aerobic and anoxic conditions**

Batch	Goal	Process used	Input parameters	Estimated parameters
A1 (non-bioaugmented)	Model calibration	Process (1)		$k_{dec\_N4\_Ox}$ , $k_{dec\_Glu\_Ox}$
		Process (2)		$k_{bio\_AS\_Ox}$
		Process (1), (4) and (6)	$k_{dec\_N4\_Ox}$ , $k_{dec\_Glu\_Ox}$ , $k_{bio\_AS\_Ox}$	$q_{bio\_AS\_Ox}$
A4 (bioaugmented)	Model calibration	Process (3)		$k_{bio\_PR1\_Ox}$ **
		Process (1), (4), (5) and (6)	$k_{dec\_N4\_Ox}$ , $k_{dec\_Glu\_Ox}$ , $k_{bio\_AS\_Ox}$ , $q_{bio\_AS\_Ox}$ , $k_{bio\_PR1\_Ox}$	$q_{bio\_PR1\_Ox}$
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)		$k_{dec\_N4\_Ax}$ , $k_{dec\_Glu\_Ax}$
		Process (8)	$k_{dec\_N4\_Ax}$ , $k_{dec\_Glu\_Ax}$	$k_{bio\_AS\_Ax}$
An2 (bioaugmented)	Model calibration	Process (7), (8) and (9)	$k_{dec\_N4\_Ax}$ , $k_{dec\_Glu\_Ax}$ , $k_{bio\_AS\_Ax}$	$k_{bio\_PR1\_Ax}$

206 \*\*  $k_{bio\_PR1\_Ox}$  was determined in our previous study [14], from the test with the pure culture (i.e. *A.denitrificans* PR1 biodegradation test) conducted in mineral medium  
 207 supplemented with SMX as the only substrate.

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### 211 3. Results and discussions

#### 212 3.1. Abiotic and sorption processes

213 Fig. 1 shows the evolution of SMX, Ac-SMX and SMX-Glu concentrations over the test period of 6 h  
214 in control test 1, revealing 1.8%, 11.4% and 11.8% removal for Ac-SMX, SMX-Glu and SMX,  
215 respectively. This suggests that abiotic processes had minor contribution to the removal of the tested  
216 compounds, in agreement with previous studies [24].

217 To investigate sorption to AS, sodium azide ( $\text{NaN}_3$ ) was used to inhibit the aerobic respiration and  
218 suppress the microbial activity of the AS (control 2). The results showed that Ac-SMX and SMX-Glu  
219 were transformed concomitantly with an increase in SMX concentration (Fig. 1), indicating that the  
220 retransformation of the parent SMX from the two human conjugates occurred even with inactivated  
221 biomass, likely via extracellular enzymes. This is in agreement with previous studies for other  
222 conjugates [25]. In terms of mass balance, supposing that all the human conjugates were converted back  
223 to SMX, there was approx.  $0.02 \mu\text{mol}$  SMX formed after 4.5 hours, while there was a removal of approx.  
224  $0.02 \mu\text{mol}$  of the two human conjugates. Therefore, no SMX removal was observed in the presence of  
225  $\text{NaN}_3$  (control 2). Since sodium azide was present at concentrations previously observed to be sufficient  
226 to inhibit the fraction of aerobic biomass [26], biotransformation of the two human conjugates could be  
227 due to the activity of facultative anaerobic bacteria, which was not sufficiently inhibited by the addition  
228 of  $\text{NaN}_3$ .

229 Due to the impossibility of determining the partitioning coefficient for SMX, the sorption fraction was  
230 assessed considering the sorption coefficient  $K_D$  obtained from previous literature. A  $K_D$  value of  $0.256$   
231  $\text{L gTSS}^{-1}$  [23] was chosen as the tests in this study were performed with fresh AS and real wastewater,  
232 which was representative of the real WWTPs where the  $K_D$  was obtained. At circumneutral pH typical  
233 of activated sludge systems, SMX is predominantly speciated as an anion ( $\text{pK}_a = 5.7$ ). Possibly due to  
234 repulsion with negatively charged sludge particles, sorption of SMX has been generally found to be  
235 limited ( $K_d < 0.4 \text{ L g}^{-1}$ ) but not negligible. Notably, the  $K_d$  value used is in agreement with other  
236 determinations in activated sludge (see, e.g. [7], [27], [23]). Sorption of the two human conjugates onto



237 AS was not considered in these experiments as no reference values of sorption coefficient were reported.  
238 Indeed, the pH of the mixed liquor in the tests was between 7.0-7.4, which is well above the  $pK_a$  of  
239 SMX-Glu and Ac-SMX ( $pK_{a2} = 2.7$  and  $5.6$ , respectively). Under these experimental conditions, Ac-  
240 SMX and SMX-Glu exist predominantly or completely as negatively charged species in the aqueous  
241 phase. Hence, negligible sorption of Ac-SMX and SMX-Glu was assumed due to their high solubility  
242 and polar nature.

### 243 **3.2. Biotransformation of SMX, SMX-Glu and Ac-SMX in bioaugmented and non-bioaugmented AS** 244 **tests**

#### 245 *Aerobic batch experiments (A1-A4)*

246 In general, biotransformation of the two human conjugates was almost complete for all the batch tests  
247 performed under aerobic conditions. This is in agreement with previous studies, showing >85% removal  
248 of the two conjugates in laboratory-scale [3] and full-scale [4] AS processes, or even fully eliminated in  
249 a pilot membrane bioreactor [28]. In the aerobic non-bioaugmented reactors (A1, A2), an increase in  
250 SMX concentration was observed in the first 4–6 hours followed by a slow decrease during the rest of  
251 the tests when biotransformation of the two human conjugates was complete (Fig. 2a and 2b). Negative  
252 SMX removal was observed, i.e. -43.1% and -63.8%, for the AS in tests A1 and A2, respectively. The  
253 decrease in Ac-SMX and SMX-Glu concentrations corresponded to increases in SMX concentrations  
254 (Fig. 2a, 2b), strongly suggesting that the two human conjugates deconjugated rapidly to form the parent  
255 compound SMX under aerobic conditions. There is relatively limited knowledge on the environmental  
256 fate and behavior of the conjugated pharmaceuticals, but these conjugates can undergo deconjugation  
257 reactions where deconjugation enzymes are present, with cleavage of the conjugated moiety, resulting  
258 in the formation of the parent pharmaceuticals [15,29].

259 For bioaugmentation of AS with PR1, experimental results obtained in the two batches A3 and A4, show  
260 a comparably high rate of SMX biotransformation in the first 4 hours, followed by a relatively lower  
261 SMX removal rate after the growth substrates were completed (Fig. 2c and 2d). Similar SMX removal  
262 was observed in A3 and A4 after 12 hours, i.e.  $92.5 \pm 1.0\%$  and  $89.4 \pm 1.5\%$ , respectively.

263 Concomitantly, complete removal of the SMX-Glu and Ac-SMX in the first 4 hours of the test was  
264 observed, which supposedly was converted back to SMX (Fig. 2c and 2d) as suggested by other studies  
265 [3,15].

#### 266 *Relative contribution of heterotrophs and ammonia oxidizing bacteria*

267 Batch tests performed in the presence of ATU showed no removal of ammonium and no formation of  
268 nitrate (Fig. 3b), suggesting nitrification was completely suppressed. There was 42 % removal of SMX  
269 after 12 hours (Fig. 3a). No appreciable differences were observed in the removal efficiency of SMX  
270 with and without ATU (Fig. 3a), suggesting negligible contribution of AOB to the biotransformation of  
271 SMX. Also, for all the remaining aerobic batch tests (A1, A2, A3, and A4) performed, no ammonium  
272 removal and no nitrate formation could be observed (Fig. S2) during the testing periods, confirming that  
273 no nitrifying activity occurred in the tested AS. Even though biotransformation of SMX in AS was  
274 previously shown to correlate with both nitrifying activity [30,31] and heterotrophic bacteria [32,33],  
275 no appreciable differences were observed in the removal efficiency of SMX with and without ATU (Fig.  
276 3a) in our study, which could be due to the fact that (i) the SMX biotransformation rate by heterotrophic  
277 aerobic degradation was reported to be much faster compared to autotrophic nitrification (e.g.  $k_{\text{bio, h}} =$   
278  $0.09 \text{ L/g}_{\text{VSS}} \text{ d}$  vs.  $k_{\text{bio, a}} = 0.01 \text{ L/g}_{\text{VSS}} \text{ d}$ ) [32]; and (ii) the possible higher abundance of heterotrophs  
279 compared to nitrifiers. Thus, heterotrophs seem to be the dominant organisms responsible for the  
280 biotransformation of SMX in the current study and the cometabolic model applied for all the batch tests  
281 could be based on only organic carbon (i.e. readily biodegradable substrates and supplemental acetate)  
282 as the primary substrates.

#### 283 *Effect of acetate addition on the biotransformation of SMX*

284 We previously showed that the presence of a biogenic substrate, e.g. acetate or succinate, provided for  
285 an 8-fold increase of SMX biotransformation kinetics by PR1 [14]. In the current study, the initial  
286 concentration of acetate was approximately 137 to 152 mg COD L<sup>-1</sup>, supplemented with preclarified  
287 wastewater to (i) the bioaugmented reactor (A4) (Fig. S8b) as a biogenic substrate to enhance the  
288 kinetics of SMX biotransformation by PR1, and (ii) the non-bioaugmented reactor (A2) (Fig. S8a) as a

289 control for comparison purposes. Upon bioaugmentation of AS with PR1 (reactor A3, Fig. 2c), with  
290 only wastewater (no acetate addition), SMX was biotransformed steadily and almost completely without  
291 any lag phase. In addition, the profiles of SMX in the tests fed with acetate were comparable to the one  
292 in the tests without acetate (Fig. S8a (A1 versus A2) and S8b (A3 versus A4)). From these observations,  
293 we hypothesized that (i) PR1 could use other available carbon sources present in wastewater as biogenic  
294 substrates to enhance the SMX biotransformation kinetics and the addition of acetate is unnecessary for  
295 the bioaugmentation with PR1; (ii) there is no enhancement effect due to acetate addition on the  
296 biotransformation of SMX by AS when fed with real wastewater. These hypotheses were also justified  
297 with the modelling results in section 3.3 and 3.4.

#### 298 *Anoxic experiments*

299 Pure culture biotransformation tests had previously shown that the *A.denitrificans* PR1 is capable of  
300 biotransformation of SMX under both aerobic [14] and anoxic conditions (data not shown). Hence the  
301 extent of SMX removal under anoxic conditions was also assessed and compared to those obtained in  
302 aerobic conditions.

303 In the two anoxic batch tests with non-bioaugmented (An1) and bioaugmented AS (An2), most of the  
304 two human conjugates were removed in the first 6 hours (Fig. 4a and 4b), which is in agreement with  
305 Stadler et al. 2015 [3] that observed >90% of Ac-SMX and SMX-Glu removals under anoxic condition.

306 For the non-bioaugmented reactor An1, the consumption of nitrate (Fig. 4c) revealed denitrifying  
307 activity, while no net SMX removal could be observed (Fig. 4a) overall. SMX concentration increased  
308 in the first 6 hours simultaneously with the deconjugation of the two human conjugates, and remained  
309 constant for the rest of the experiment. This is opposed to what was observed in denitrifying AS [7] and  
310 denitrifying MBBR sludge [34,35]. In contrast, in the bioaugmented reactor (An2), a slight decrease in  
311 SMX concentration was observed after the retransformations of the two human conjugates had  
312 completed (after 4 hours) (Fig. 4b). This suggests a biotransformation of SMX associated with the  
313 activity of the bioaugmented strain PR1, but at a rather slow rate.

### 314 3.3. *Model-based assessment of biotransformation kinetics*

315 Experimental data obtained in the batches A1, A4 and An1, An2 was used for the estimation of the  
316 biotransformation rate constants for AS and the *A. denitrificans* PR1 under aerobic and anoxic  
317 conditions. Predicted dissolved concentration profiles of SMX and its two human conjugates during  
318 batch experiments are compared with measured data and shown in Fig. 2a, 2c, Fig. 4a and 4b. The  
319 estimated parameters are summarized in Table 4. The model predictions were evaluated using the R-  
320 squared ( $R^2$ ) coefficient, shown in Fig. S3-S5, and summarized in Table S4 (SI). Confidence intervals  
321 were obtained by the estimation of standard deviations for a level of confidence of 95% (Fig. S3-S5,  
322 SI).

#### 323 *Kinetics of deconjugation*

324 Deconjugation kinetics of the two conjugates could be described with pseudo-first order kinetics, in  
325 processes for aerobic (1) and anoxic (9) conditions. For the conjugated Ac-SMX, fitting of measured  
326 data resulted in  $k_{Dec}$  values of  $8.9 \pm 0.53 \text{ L gTSS}^{-1} \text{ d}^{-1}$  for aerobic conditions, which was almost 2-fold  
327 higher than anoxic conditions ( $5.30 \pm 0.21 \text{ L gTSS}^{-1} \text{ d}^{-1}$ ) (Table 4). These data agree well with values  
328 reported in literature for  $k_{Dec}$  of 5.9-7.6  $\text{L gTSS}^{-1} \text{ d}^{-1}$  [7,36] under aerobic conditions or 7.9  $\text{L gTSS}^{-1} \text{ d}^{-1}$   
329 [7] under anoxic conditions. No difference in the rate constants of SMX-Glu under aerobic and anoxic  
330 conditions was obtained ( $4.76 \pm 0.4$  and  $4.74 \pm 0.31 \text{ L gTSS}^{-1} \text{ d}^{-1}$ , respectively). No data for the  
331 biotransformation rate coefficients of SMX-Glu were available in literature for comparison. Good  
332 agreement between experimental data and model simulations for Ac-SMX and SMX-Glu was shown,  
333 as confirmed by high  $R^2$  coefficients ( $\geq 0.98$ ), indicating that pseudo-first order equations describe well  
334 the biotransformation kinetics of the two human conjugates. These results suggest that (i) deconjugation  
335 rate constants are well above 1  $\text{L gTSS}^{-1} \text{ d}^{-1}$ , thus indicating high degradability for conjugates; and (ii)  
336 deconjugation kinetics depend on redox conditions for Ac-SMX only, being faster under aerobic  
337 conditions.

338 *Kinetics of SMX biotransformation under aerobic conditions*

339 The removal of SMX under aerobic conditions was predicted using different mathematical models for  
340 comparison (i) a pseudo-first order kinetic model; and (ii) cometabolic models. Fig. 2 and Fig. S6 (SI)  
341 compare predicted and measured concentrations of SMX in the batches A1-A4, using a cometabolic  
342 model and a pseudo first order model, respectively. According to the data plotted in these figures, the  
343 prediction of SMX biotransformation was significantly improved by adopting the cometabolic model  
344 ( $R^2$  ranged from 0.79 to 0.99, Table S4, SI) compared to pseudo-first order biotransformation model ( $R^2$   
345 ranged from 0.044 to 0.94). These results: (i) show that the cometabolic model was able to consistently  
346 describe the experimental data, with measured concentrations that fall well within the 95% confidence  
347 interval (Fig. S3, S4, SI), making the cometabolic model the relevant choice for description of the  
348 removal of SMX; (ii) support our hypothesis of the deconjugation of Ac-SMX and SMX-Glu results in  
349 the formation of parent compound SMX, which likely explains the previously observed  
350 variability/negative SMX removal efficiencies in biological treatment. In this study, we also simulated  
351 two other scenarios for the fate of SMX, i.e.: (i) biotransformation of the two human conjugates leading  
352 to the formation of a compound different from SMX (model simulations presented as blue dashed lines  
353 in Fig. 2 and 4); and (ii) all of the Ac-SMX and SMX-Glu are converted back to parent SMX, but no  
354 SMX is biodegraded, and the model simulation is presented as black dashed lines in Fig. 2 and Fig. 4.  
355 However, the model simulations in these scenarios were far different from the respective observed  
356 concentrations of SMX in all the batch experiments (Fig. 2 and 4), indicating that neither situation is  
357 applicable in the current study.

358 According to our simulation results, a SMX biotransformation rate constant  $k_{\text{bio,AS}}$  of  $0.47 \pm 0.03$  L  
359  $\text{gTSS}^{-1} \text{d}^{-1}$  and a cometabolic biotransformation rate constant  $q_{\text{bio\_AS}}$  of  $7.97 \pm 0.51$  L  $\text{gTSS}^{-1} \text{d}^{-1}$  (Table  
360 4) were obtained by AS under aerobic conditions. Previous studies reported  $k_{\text{bio,SMX}}$  of  $0.14 - 0.41$  L  
361  $\text{gTSS}^{-1} \text{d}^{-1}$  [7,27,32,37,38], in agreement with our  $k_{\text{bio,AS}}$  result.

362 For the bioaugmented AS, test A4 – with acetate supplementation, values of measured SMX  
363 concentration data plotted as a function of time elapsed show comparably high biotransformation rate in

364 the first 4-6 hours (when primary substrate was available), followed by lower removal rate during the  
365 remaining time (following primary substrate depletion) (Fig. 2d). By using the obtained parameters  $k_{\text{bio,AS}}$ ,  
366  $q_{\text{bio\_AS}}$  from the A1 test,  $k_{\text{bio,PR1}}$  ( $56.20 \pm 3.70 \text{ L gTSS d}^{-1}$ ) from our previous study [14], fitting of measured  
367 data in the substrate depletion phase using cometabolic model as described in Table 2 resulted in  
368 estimation of  $q_{\text{bio,PR1}} = 528.39 \pm 6.78 \text{ L gTSS}^{-1} \text{ d}^{-1}$ . This  $q_{\text{bio,PR1}}$  value is consistent with rate constants  
369 ( $k_{\text{bio,PR1}} = 445.6 - 570.1 \text{ L gTSS}^{-1} \text{ d}^{-1}$ ) previously obtained with PR1 when acetate was supplemented as  
370 a biogenic substrate to enhance the biotransformation rate of SMX in pure culture biodegradation tests  
371 [14]. Higher biotransformation kinetics of SMX by PR1 ( $q_{\text{bio, PR1}}$  and  $k_{\text{bio, PR1}}$ ), compared to the  
372 retransformation kinetics of the two human conjugates ( $k_{\text{Dec}}$ ), likely lead to the observation of no increase  
373 in SMX concentration in bioaugmented AS tests (A3 and A4), differently than what was observed in non-  
374 bioaugmented AS tests (A1 and A2).

375 In general, two different kinetic rates of the removal of SMX are obtained for AS as well as for *A.*  
376 *denitrificans* PR1: a fast rate  $q_{\text{bio}}$  when primary substrate was available and a slower rate  $k_{\text{bio}}$  when  
377 primary substrate was depleted. These results can likely explain the two patterns of SMX  
378 biotransformation observed in bioaugmented batch tests A3 and A4 (Fig. 2c and 2d). As a result of  
379 cometabolism, SMX removal was enhanced in the presence of primary substrates (as characterized by  
380  $q_{\text{bio}}$ ), with a subsequent decrease of biotransformation kinetics upon primary substrate limitation  
381 (characterized by the  $k_{\text{bio}}$ ) at the end of the A3 and A4 tests (Fig. 2c and 2d).

382 Also, the significant differences between  $k_{\text{bio}}$  and  $q_{\text{bio}}$  imply that growth substrates (readily biodegradable  
383 substrates) availability can substantially impact the removal of SMX as a result of cometabolism. In fact,  
384 typically present in wastewater at very low concentrations ( $\text{ng L}^{-1}$  to  $\mu\text{g L}^{-1}$ ), micropollutants are unable  
385 to support cell replication and primary substrates (e.g. readily biodegradable carbon sources or  
386 ammonium) are essential for biomass growth and to induce enzymes for assimilation or co-factors for  
387 biotransformation [39]. As wastewater is a complex medium where not only micropollutants but also  
388 organic matter and nutrients are present, which could be degraded simultaneously by AS, cometabolism  
389 kinetics could be suitable to predict the behavior of micropollutants in real WWTPs.

390 In addition, the SMX biotransformation rate constants by *A. denitrificans* PR1, e.g.  $q_{\text{bio,PR1}}$  in the presence

391 and  $k_{\text{bio,PR1}}$  in the absence of growth substrates, are three and two orders of magnitude higher, respectively,  
392 than that estimated for AS, confirming a specialized biotransformation capability by PR1 in comparison  
393 to the mixed AS community. Thus, bioaugmentation of AS with PR1 substantially enhanced the  
394 biotransformation rate of SMX.

#### 395 *Kinetics of SMX biotransformation under anoxic conditions*

396 SMX retransformation and removal under anoxic conditions can be predicted using pseudo-first order  
397 kinetics (processes (7) – (9)), thereby allowing for the estimation of  $k_{\text{bio,Ax}}$  ( $\text{L gTSS}^{-1} \text{d}^{-1}$ ). In Fig. 4a-b,  
398 simulated and corresponding measured concentrations of the three compounds in An1 and An2 batch  
399 experiments are plotted. High  $R^2$  values ( $\geq 0.98$ ) were obtained, and measured concentrations always fell  
400 within the 95% confidence interval (Fig. S5, SI), indicating that the pseudo-first order model was able  
401 to predict the fates of SMX and the two human conjugates obtained in the anoxic experiments. The  
402 estimated  $k_{\text{bio,Ax}}$  for SMX biotransformation are  $13.57 \pm 2.10$  and  $0 \text{ L gTSS}^{-1} \text{d}^{-1}$  for *A. denitrificans* PR1  
403 and AS, respectively (Table 4). The latter value is in contrast with other studies. Plósz et al. [7] obtained  
404 a SMX biotransformation rate constant of  $0.41 \text{ L gTSS}^{-1} \text{d}^{-1}$  under anoxic conditions with AS. In other  
405 studies, values of  $0.1$  and  $0.05 \text{ L gTSS}^{-1} \text{d}^{-1}$  were reported for SMX biotransformation rate constants of  
406 heterotrophic denitrification and autotrophic denitrification, respectively [32]. Torresi et al. [34]  
407 reported a rate constant  $k_{\text{bio}}$  of  $0.1 \pm 0.1 \text{ L gTSS}^{-1} \text{d}^{-1}$  and  $q_{\text{bio}}$  of  $1.7$  and  $3.2$  for SMX biotransformation  
408 in a post-denitrification MBBR system dosed with methanol and ethanol, respectively. The obtained  
409 results suggest that, upon bioaugmentation to AS, PR1 could also be able to degrade SMX under anoxic  
410 conditions but at a significantly lower rate as compared to aerobic conditions – decreasing by 4-fold in  
411 terms of the rate constant ( $k_{\text{bio,PR1}}$ ) under anoxic conditions as compared to aerobic conditions (Table 4).

412 In the current study, we also provided a detailed description of SMX removal in AS processes when  
413 assessing the biotransformation of the parent compound and the deconjugation of the two major human  
414 conjugates (Ac-SMX and SMX-Glu) back to SMX. SMX formation from the deconjugation of the two  
415 human conjugates was experimentally observed and confirmed by model-based predictions. Ac-SMX  
416 and SMX-Glu were detected at levels that are comparable to the SMX concentrations in WWTPs [6,40]

417 (see also Table S3, SI). Significant retransformation of SMX can take place in WWTPs at a higher rate  
418 compared to its removal rate (Table 4), resulting in the sometimes negative or varied SMX removal that  
419 have been observed in many studies. It implies that deconjugation of human conjugates should be taken  
420 into account to thoroughly understand the fate and removal of SMX during wastewater treatment.

421 In general, the results of these tests highlight the potential application of *A. denitrificans* PR1 for  
422 bioaugmentation for SMX removal in WWTPs. One criterion for a successful bioaugmentation is the  
423 metabolically active inoculum of a microorganism or consortium. Inability of the inoculated strains to  
424 degrade the xenobiotic chemicals once augmented into AS has been reported [41]. One explanation  
425 given for such failure in bioaugmentation was the presence of alternative readily biodegradable  
426 substrates [41]. In our experiments, enhancement of SMX biotransformation upon bioaugmentation of  
427 AS with PR1 was observed. Upon bioaugmentation of AS with *A. denitrificans*, without lag phase, a  
428 fast biotransformation of SMX was observed at rates similar to those obtained in pure culture  
429 biodegradation tests when acetate was supplemented as biogenic substrate. In addition, the SMX  
430 reaction rate constant and cometabolic biotransformation rate of PR1 were about two orders of  
431 magnitude higher than the kinetics of AS, regardless of the presence of additional acetate. The fact that  
432 the strain was able to use the complex substrates present in real wastewater to stimulate the activity and  
433 provide energy for growth and maintenance, suggests that PR1 has a great potential to survive in AS  
434 communities upon bioaugmentation. Overall, bioaugmentation with PR1 appears to be a feasible  
435 solution for enhancing SMX removal in wastewater, while further studies should focus on long-term  
436 biotransformation activity and stability of the bioaugmentation strain in wastewater systems in order to  
437 make bioaugmentation applicable.

### 438 **3.4. Model validation**

439 The models for retransformation of SMX from the two human conjugates and cometabolic  
440 biotransformation of SMX were validated using the two sets of experimental results, A2 and A3, for the  
441 non-bioaugmented and bioaugmented cases, respectively (Fig. 2b and 2c). The set of estimated parameter  
442 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



443 to predict the behaviour of the three compounds (SMX, Ac-SMX and SMX-Glu) in the reactors A2 and  
444 A3, providing measured data independent from those used for model identification. Measured and  
445 predicted concentrations were compared and  $R^2$  was calculated to determine the extent of correlation.  
446 Good agreement between the experimental data and model simulations could be observed with high  $R^2$   
447 ( $\geq 0.95$ , Table S4). This indicates the applicability of the model towards the prediction of the fate of SMX  
448 and human metabolite biotransformation by both AS and PR1 (Fig. 2b and 2c), even in the presence of  
449 an externally dosed carbon source (in this case acetate).

450 As cometabolic biotransformation depend on the readily biodegradable growth substrates,  $S_s$  (mgCOD  
451  $L^{-1}$ ), the addition of acetate to AS would affect the biotransformation of SMX. From our previous study  
452 [14] as well as the modelling result of the bioaugmentation test A4 (section 3.3.2), there is no doubt  
453 that acetate is a biogenic substrate to enhance the SMX biotransformation by *A. denitrificans* PR1, i.e.  
454 primary substrate for the cometabolism of SMX. For the non-bioaugmented activated sludge (A2), we  
455 hypothesized above (section 3.2.1.2) that there is no enhanced effects of acetate on the biotransformation  
456 of SMX by AS. To test this hypothesis, for modelling of SMX biotransformation of non-bioaugmented  
457 AS (batch A2 – with the supplementation of acetate), we tested, (i) both acetate and other readily  
458 biodegradable substrates that are present in wastewater (expressed as sCOD); (ii) only readily  
459 biodegradable substrates that are present in wastewater (expressed as sCOD) were considered as the  
460 primary substrates ( $S_s$ ) in the cometabolic model to enhance the SMX biotransformation by AS.  
461 However, only the latter option gave good fitting between measured and model-based prediction (Fig.  
462 S7, SI vs. Fig. 2b), suggesting that the readily biodegradable substrates that are present in wastewater  
463 (expressed as sCOD) acted and were sufficient as primary substrates for the cometabolism of SMX by  
464 AS. Acetate was measured in the wastewater and it was typically below 7 mg/L, therefore the  
465 microorganisms were probably not particularly adapted to it. Müller et al. [33] observed that SMX  
466 cometabolism with acetate by AS occurred only after a sufficient adaptation time, meaning that the  
467 supplementation of additional acetate might have still enhanced the SMX further if sufficient adaptation  
468 time was allowed, although little is known about which easily biodegradable compounds are used as  
469 primary substrates by the AS community. Although the  $R^2$  calculated for the SMX in the A2 test (Fig.

470 2b) was equal to 0.79, the difference between measured and predicted SMX concentrations were still  
471 within the standard deviations of the measured concentrations and falls in between confidence interval  
472 boundaries (Fig. S3), making the cometabolic model term still relevant.

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

#### 482 **4. Conclusions**

483 In this study, six different batch tests with non-bioaugmented and bio-augmented (with *A. denitrificans*  
484 PR1) AS, operated under different redox conditions and primary substrate addition levels, were used for  
485 in-depth assessment of SMX removal in combination with its two main human conjugates (Ac-SMX and  
486 SMX-Glu). The extent of SMX removal varied depending on the experimental conditions, and process  
487 models were used for interpretation of experimental results and determination of biotransformation  
488 kinetics. The results from the experimental and model-based predictions show that the conversion of Ac-  
489 SMX and SMX-Glu back to parent SMX was confirmed in activated sludge, whereby deconjugation in  
490 non-bioaugmented AS was significantly faster than SMX biotransformation. This likely explains the  
491 previously observed variability of SMX removal efficiencies, including net formation of SMX, in full-  
492 scale biological WWTPs. The biotransformation of SMX and the deconjugation of Ac-SMX clearly  
493 depend on the redox conditions, with the highest removal occurring under aerobic conditions.  
494 Deconjugation of SMX-Glu was however independent of redox conditions. Also, cometabolic models  
495 were successfully used to predict the biological transformation kinetics of SMX in both bioaugmented

496 and non-bioaugmented reactors under different test conditions. Estimation of kinetic parameters allowed  
497 for the assessment of the success of the bioaugmentation strategy and for the identification of best  
498 conditions for its applicability. Furthermore, estimated kinetic parameters obtained from this study could  
499 be integrated in AS models to predict the fate of SMX during the biological treatment in WWTPs. Overall,  
500 bioaugmentation of AS with *A. denitrificans* strain PR1 led to enhanced (100-fold) biotransformation  
501 kinetics of SMX compared to the non-bioaugmented AS, within a complex carbon environment found at  
502 a WWTP without an addition of another C-source (acetate) as specific substrate for the biotransformation  
503 of SMX. These results prospect the use of *A. denitrificans* PR1 for bioaugmentation as a feasible and  
504 efficient option to improve SMX elimination in WWTPs.

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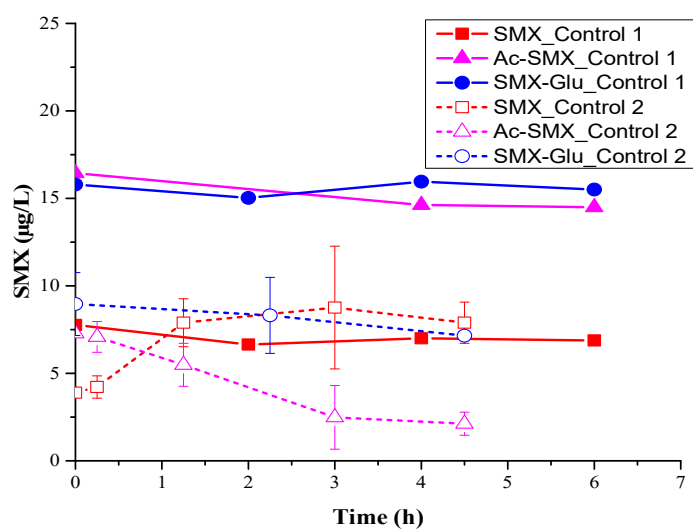
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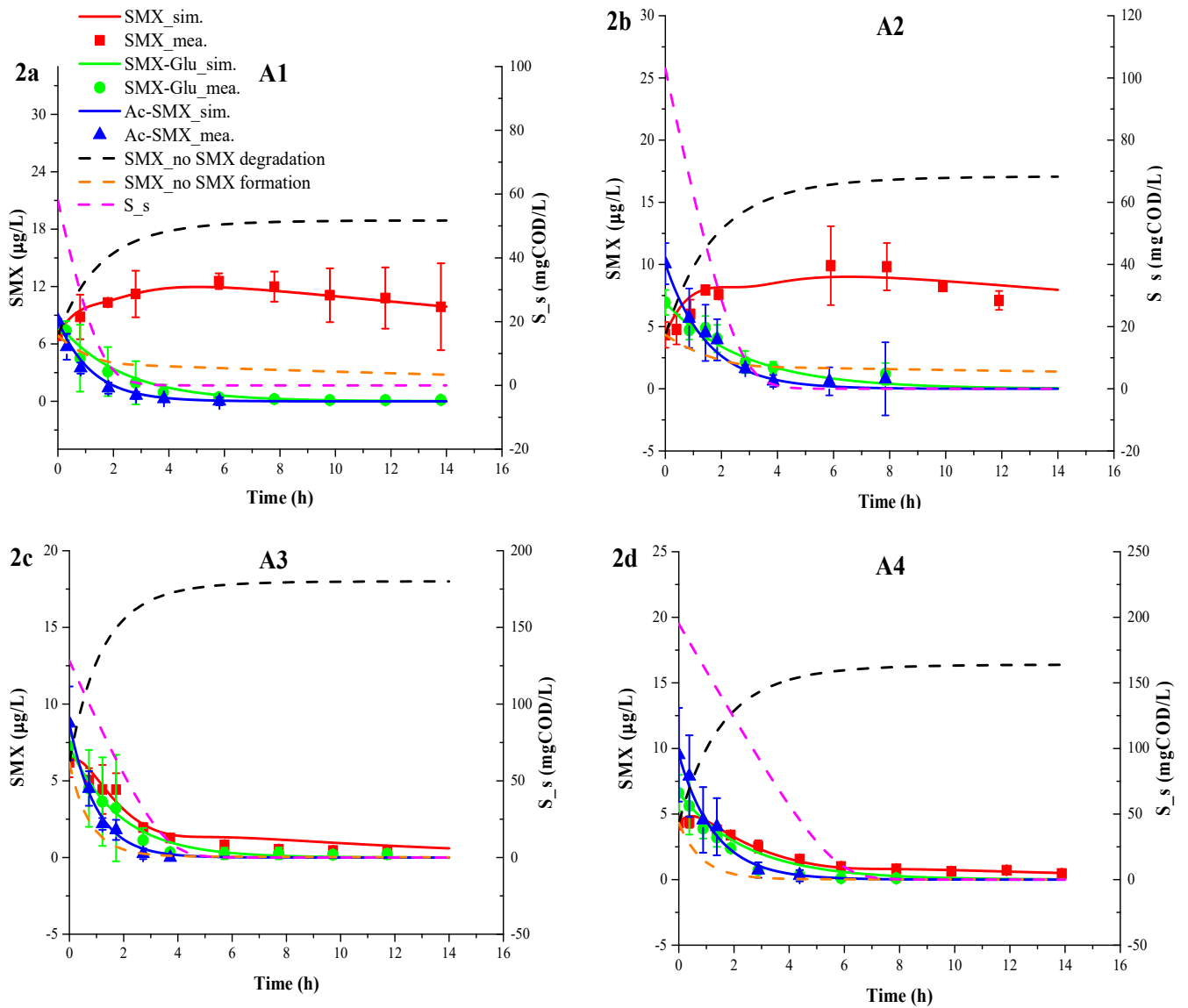


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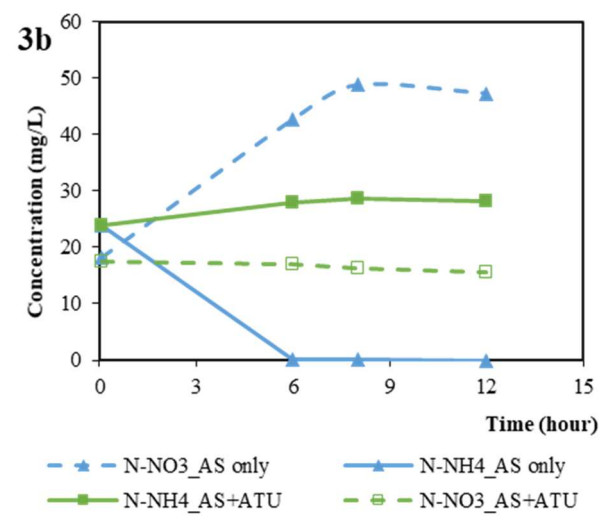
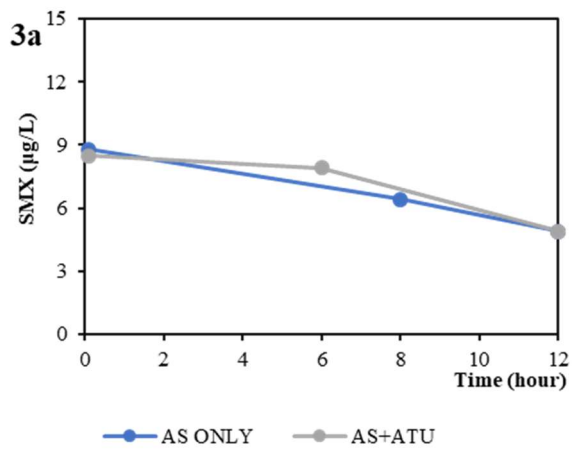
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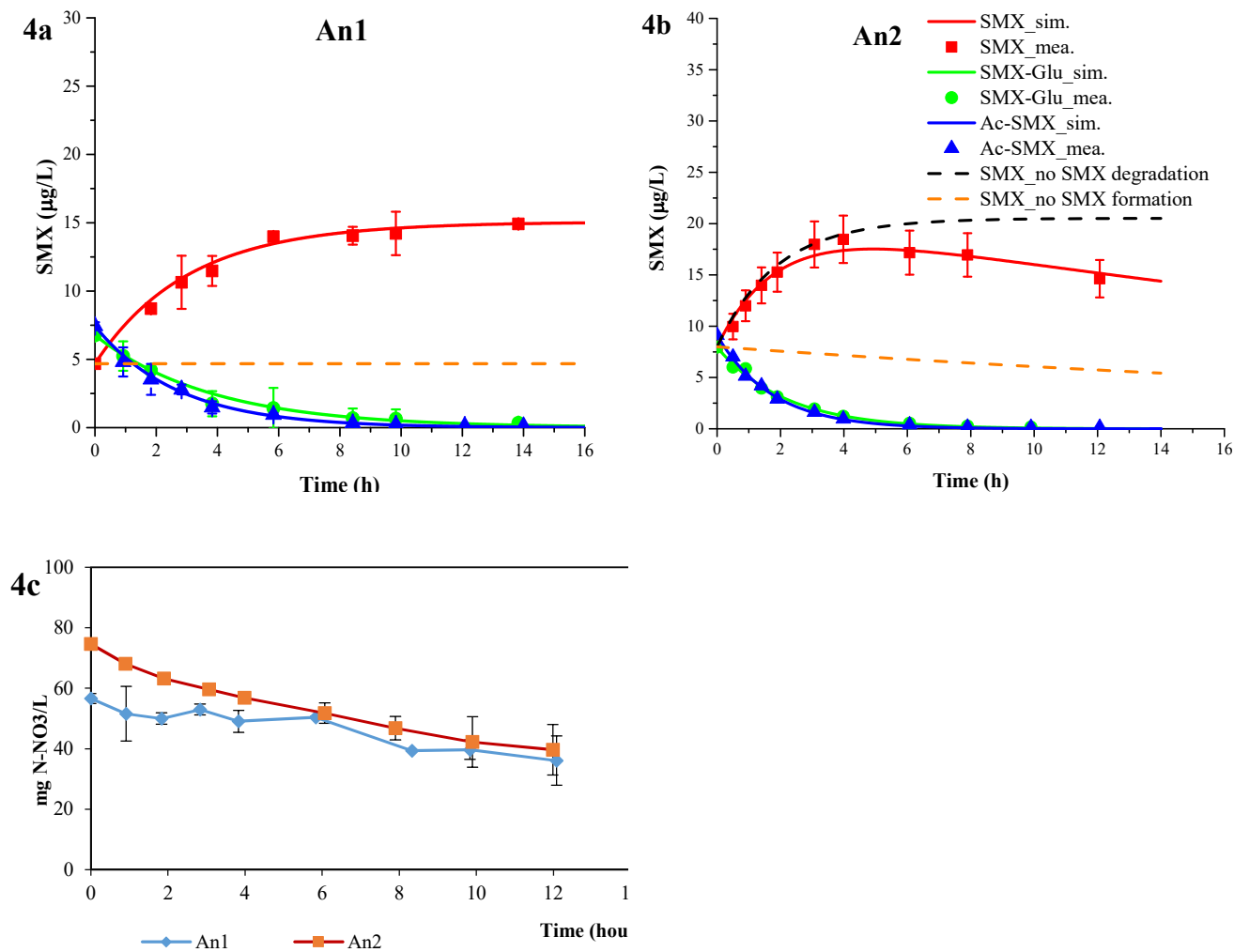
**Fig. 1. Measured concentrations of SMX, Ac-SMX, and SMX-Glu as a function of time for the control batch tests, i.e. control 1-with Milli-Q water (continuous lines), control 2-with  $\text{NaN}_3$  (dashed lines) as an inhibitor. Error bars indicate the standard deviations for duplicates**



**Fig. 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.**



**Fig. 3. Effect of ATU inhibition on removal of SMX (3a) and nitrogen (3b)**



**Fig. 4. Illustration of measured concentrations of SMX, N4-acetyl-SMX, and SMX-N1-Glucuronide (markers) and simulated (lines) as a function of time 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. Nitrate consumption for the batch tests is also shown (4c). Error bars indicate the standard deviations for duplicates.**

**Table 4. Model parameters and information of 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		N4--SMX	
			AS	PR1	AS	PR1
<b>Aerobic</b>						
$k_{Dec,Ox}$	Aerobic biotransformation rate coefficient for the human conjugates, $C_{CJ}$	L g TSS <sup>-1</sup> d <sup>-1</sup>	4.76 ± 0.38 (n.a)	-	8.9 ± 0.53 (5.9-7.6 [36]) (6.8 <sup>[7]</sup> )	-
$k_{bio,Ox}$	Aerobic biotransformation rate coefficient for the parent compound, $C_{LI}$	L g TSS <sup>-1</sup> d <sup>-1</sup>	-	-	-	-
$q_{bio,Ox}$	Aerobic cometabolic-biotransformation rate constant for parent compound, $C_{LI}$	L g TSS <sup>-1</sup> d <sup>-1</sup>	-	-	-	-
<b>Anoxic</b>						
$k_{Dec,Ax}$	Anoxic biotransformation rate coefficient for the human conjugates, $C_{CJ}$	L g TSS <sup>-1</sup> d <sup>-1</sup>	4.74 ± 0.31 (n.a)	-	<b>5.30 ± 0.21</b> (7.9 <sup>[3]</sup> )	-

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$k_{bio,Ax}$	Anoxic biotransformation rate coefficient for the parent compound, $C_{LI}$	$L\ g\ TSS^{-1}\ d^{-1}$	-	-	-	-
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n.a.: not available

## Supplementary Material Section

### **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**

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(14 pages, 5 Tables, 8 Figures)



## **S1. Sample preparation and analytical methods for the concentration of sulfamethoxazole, the two human metabolites and biodegradation metabolites with HPLC-LC-MS/MS**

### ***Sample preparation***

The following SPE procedure was based on a previously published method for the analysis of sulfonamides in natural waters [1]. The sample was filtered through nylon syringe filter 0.2  $\mu\text{m}$  (Whatman). The supernatant was stored in 10 mL glass vials until analysis (within 2 weeks). Before performing SPE, the sample aliquot was added with  $\text{Na}_2\text{EDTA}$  solution as a complexing agent, and was spiked with surrogate standards d4-N<sub>4</sub>-acetyl-sulfamethoxazole, sulfamethoxazole-d4-N<sub>1</sub>-glucuronide, sulfamethoxazole-d4 each at 500 ng/L, 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 cartridge (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  $\sim 5$  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  $\sim 5$  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  $\mu\text{L}$  in a solvent mixture of MilliQ-water:methanol (9:1). The extract was transferred to an amber autosampler vial, and stored at  $-20^\circ\text{C}$  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 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  $\mu\text{m}$ ), Waters)) at  $40^\circ\text{C}$  using an injection volume of 20  $\mu\text{L}$ . 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), cone voltage.

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<sub>2</sub>) as 22 psi and drying gas flow rate as 11 L/min to achieve the highest sensitivity. Ultra high-purity Argon (Ar) was used as collision gas. High purity nitrogen was used as sheath, auxiliary and sweep gas.

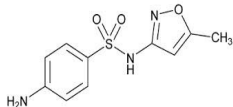

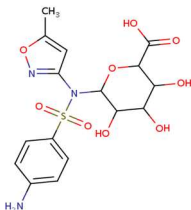
MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in Table S1. The first transition corresponds to the most abundant and was used for quantification and the second one for confirmation purposes.

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

**Table S5. MS/MS parameters for the analysis of target analytes by MRM positive ionization mode**

Target compounds	R <sub>t</sub> (min)	Precursor ion [M+H] <sup>+</sup>	MRM1		MRM2	
			Collision energy (eV)	Production ion	Collision Energy (eV)	Production ion
Sulfamethoxazole	5.84	254.1	15	156	20	92
N <sub>4</sub> -acetyl-sulfamethoxazole	6.81	296.3	25	134.1	18	198.1
sulfamethoxazole-N <sub>1</sub> - 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 <sub>4</sub> -acetyl-sulfamethoxazole	6.82	300.3	25	138.2	18	202.2
sulfamethoxazole-d4-N <sub>1</sub> - 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 S6. 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 concentration ( $\mu\text{g L}^{-1}$ )
Sulfamethoxazole		Antibiotic	0.89 <sup>[2]</sup>	2.4 <sup>[3]</sup>	$pK_{a1} = 1.8^{[4]}$ $pK_{a2} = 5.7^{[4]}$	$0.87 \pm 0.75$
Ac-acetyl-sulfamethoxazole		Antibiotic TP	N.F	N.F	$5.6 \pm 0.5^{[4]}$	$0.98 \pm 0.2$
Sulfamethoxazole- $N_1$ -glucuronide		Antibiotic TP	1.21	N.F	$2.7 \pm 0.5^{[4]}$	n.d.

n.d. : not determined

## S2. Chelas Wastewater Treatment Plant (Lisbon, Portugal)

Municipal WWTP Chelas was designed to receive about 52500 m<sup>3</sup> 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-denitrification 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 S3. The average treatment performance of Chelas WWTP was 90% removal of N-NH<sub>4</sub><sup>+</sup>, 60% removal of N-NO<sub>3</sub><sup>-</sup>.

**Table S7. Primary effluent wastewater characteristics**

	TCOD (mg L <sup>-1</sup> ) (n=23)	sCOD (mg L <sup>-1</sup> ) (n=23)	BOD <sub>5</sub> (mg L <sup>-1</sup> ) (n=13)	N-NH <sub>4</sub> <sup>+</sup> (mg L <sup>-1</sup> ) (n=76)	SMX (µg L <sup>-1</sup> ) (n=3)	Ac-SMX (µg L <sup>-1</sup> ) (n=3)
<b>Range</b>	126-500	50-139	33-143	40-53	0.52 – 1.73	0.78 -1.17
<b>Mean ± std</b>	258 ± 97	99 ± 22	91 ± 37	40 ± 8	0.87 ± 0.75	0.98 ± 0.20

TCOD: total COD; sCOD: soluble COD

### S3. Model calibration procedure

For model calibration, the  $k_{\text{bio,AS}}$  value was approximated based on process (2) (Table 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 (Fig. 2a).  $K_D$  values (shown in Table 2) were used to assess the sorption fraction, while the constant value  $X_{\text{AS}}$  in Table 2 represent activated sludge (AS) biomass concentration. Process (1), (4) and (6) (Table 2) allows estimation of  $q_{\text{bio,AS}}$ , using the  $k_{\text{bio,AS}}$  value obtained above, the  $K_D$  value (shown in Table 2), and a constant value for  $X_{\text{AS}}$ .

Data obtained from the bioaugmented test A4 was used to determine the cometabolic biotransformation rate constant of *A. denitrificans* PR1, i.e.  $q_{\text{bio,PR1}}$ . Biotransformation of SMX in this experiment was attributed to the activity of both AS and the strain PR1 (Table 2, process (1), (4), (5) and (6)) and characterized by the biotransformation rate constants of AS ( $k_{\text{bio,AS}}$  and  $q_{\text{bio,AS}}$ ) and of PR1 ( $k_{\text{bio,PR1}}$  and  $q_{\text{bio,PR1}}$ ). Biotransformation kinetic associated with AS were previously estimated through calibration against A1 test results, while  $k_{\text{bio,PR1}}$  was derived from our previous study under primary substrate limitation [5].

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

Experimental results from An1 were used to determine the retransformation rate, i.e.  $k_{\text{Dec,Ax}}$  and SMX biotransformation kinetics of AS, i.e.  $k_{\text{bio,AS}}$ , under anoxic conditions using processes (7) and (8) (Table 2). The anoxic  $k_{\text{bio,AS}}$  was then used as an input for predicting An2 results and estimating the biotransformation rate coefficient of strain PR1 ( $k_{\text{bio,PR1}}$ ) using processes (7), (8) and (9) (Table 2).

#### S4. Standard deviation calculated for average samples

The standard deviations of the measured concentrations of the three target compounds, e.g. sulfamethoxazole, N<sub>4</sub>-acetyl-sulfamethoxazole, and SMX-N<sub>1</sub>-Glucuronide were calculated by STDEV function in Excel that uses the following formula:

$$\sqrt{\frac{\sum (x - \bar{x})^2}{(n-1)}} \quad (\text{Eq. S1})$$

where x is the sample mean of the duplicate of two tests and n is the sample size, in this study n=2.

**Table S8. Goodness of the fit (R<sup>2</sup>) of the models used in this study**

Test	Compound	Biotransformation		Retransformation	
		Pseudo-first kinetic	order	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 S5. Parameters of the ASM model [6] used and calibrated in this study**

Parameter	Definition	Values	Unit
μ <sub>H</sub>	Specific growth rate of heterotrophs	Calibrated	day <sup>-1</sup>
Y <sub>H</sub>	Yield coefficient for heterotrophs	0.67	g cell COD formed (g COD oxidized) <sup>-1</sup>

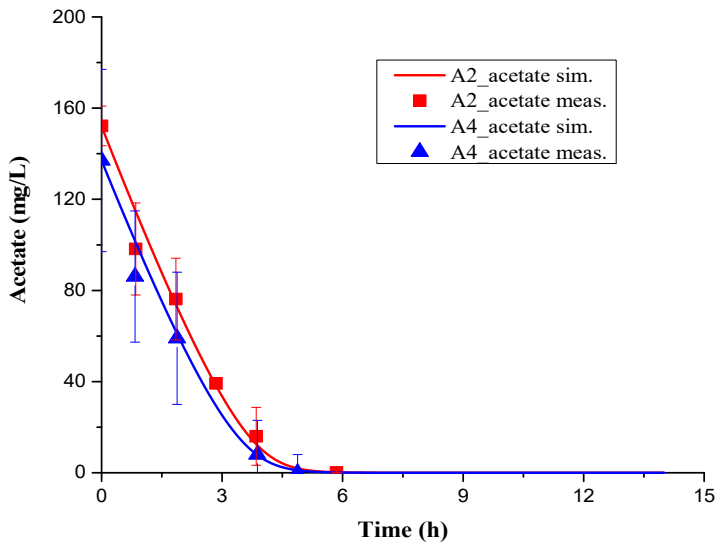


Fig. S5. Measured and simulated acetate, expressed as sCOD (mg/L) for aerobic batch tests (A1): non-bioaugmented AS with supplementation of acetate; and (A4): bioaugmented AS with supplementation of acetate. Error bars indicates standard deviation for duplicates

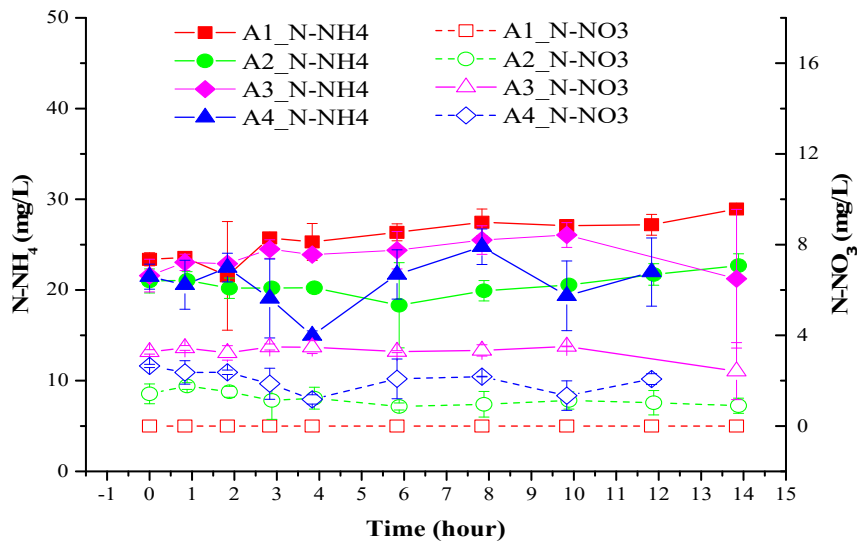
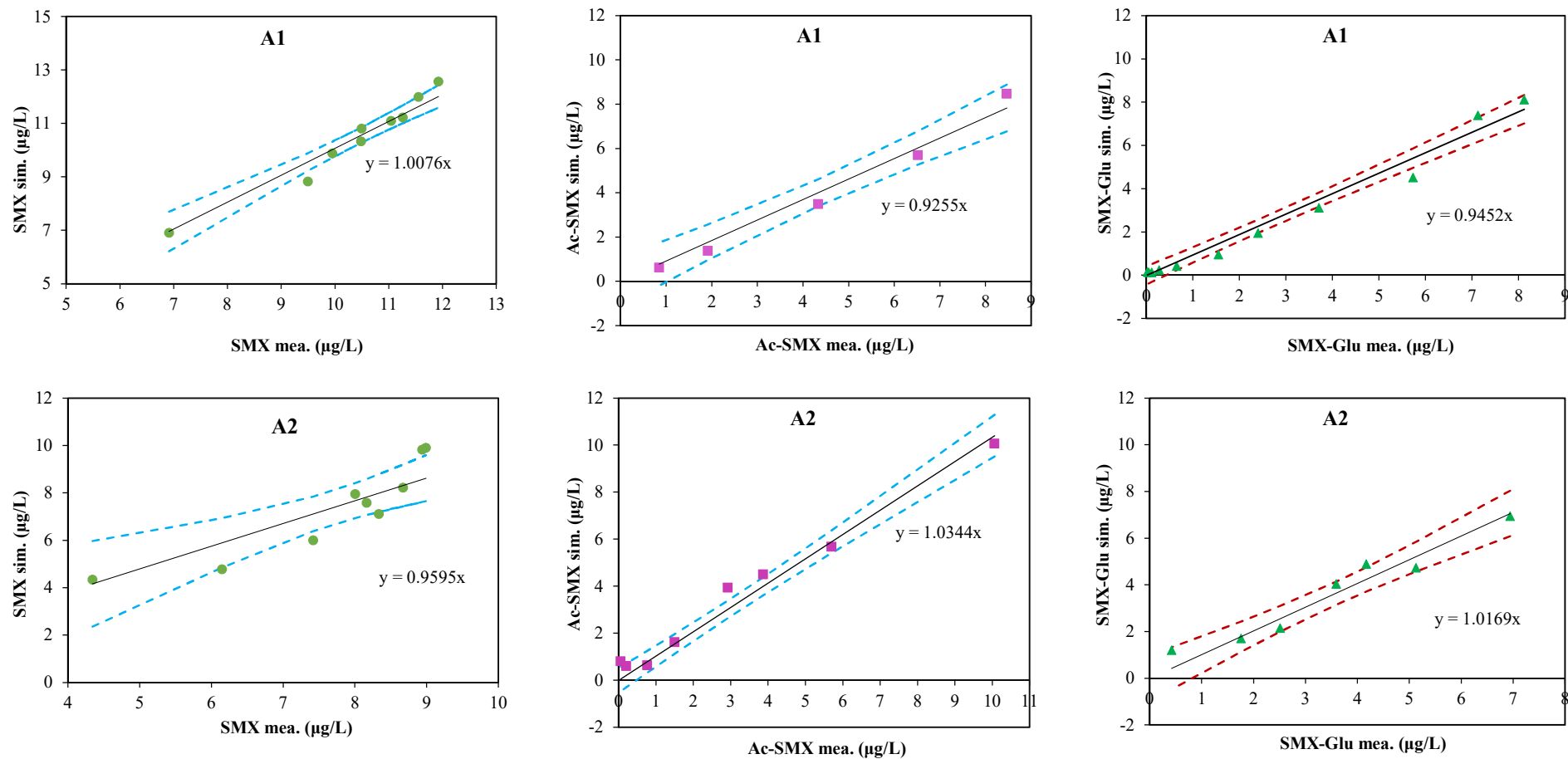
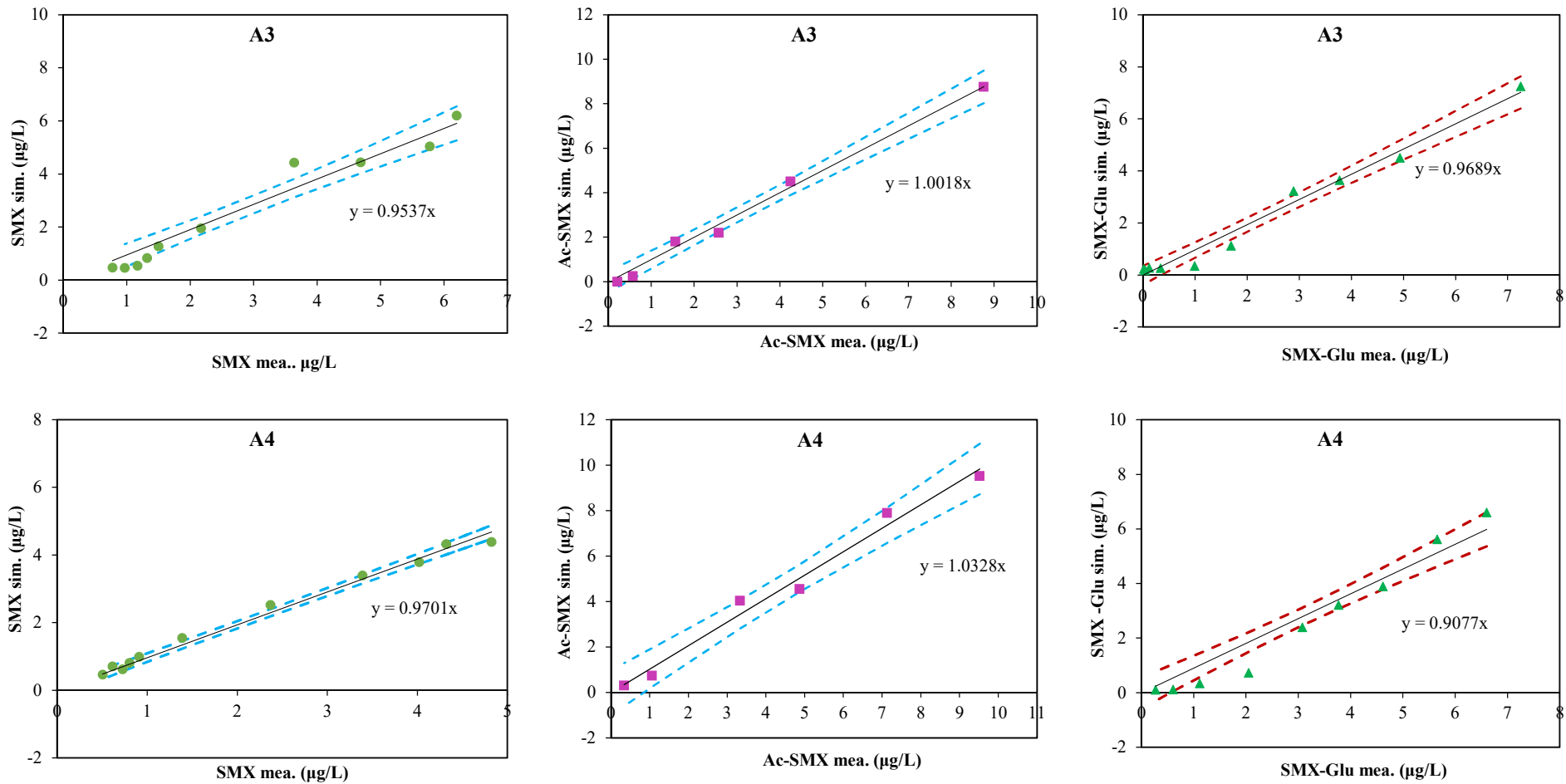


Fig. S2. Evolution of ammonium concentration for aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplementation of acetate as additional C-source; (A3): bioaugmented activated sludge with *A. denitrificans* PR1 test; and (A4): bioaugmented activated sludge with *A. denitrificans* supplemented with acetate test. Error bars indicate standard deviations for duplicates.



**Fig. S3. 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.



**Fig. S4. 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.



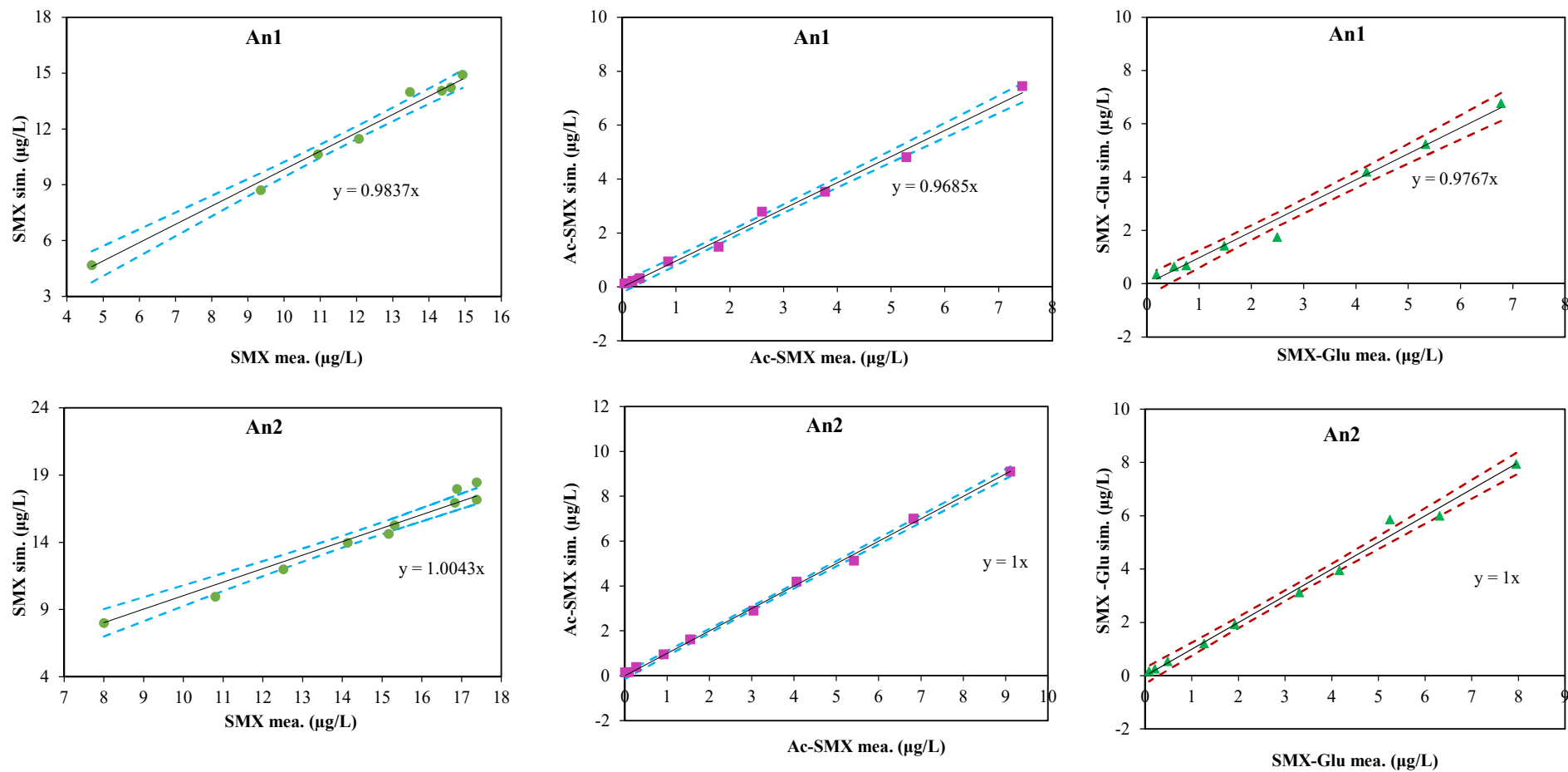
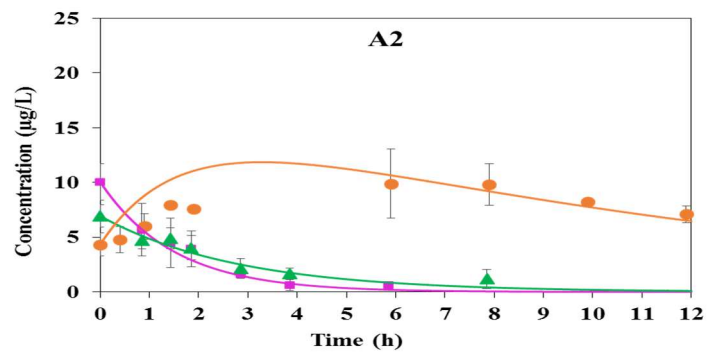
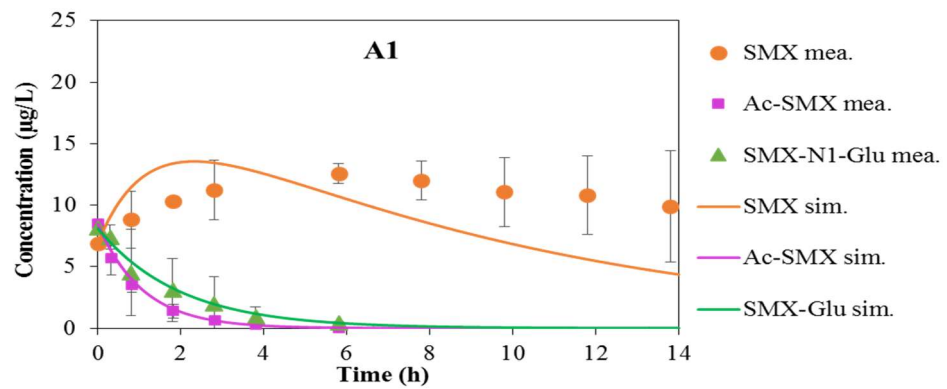
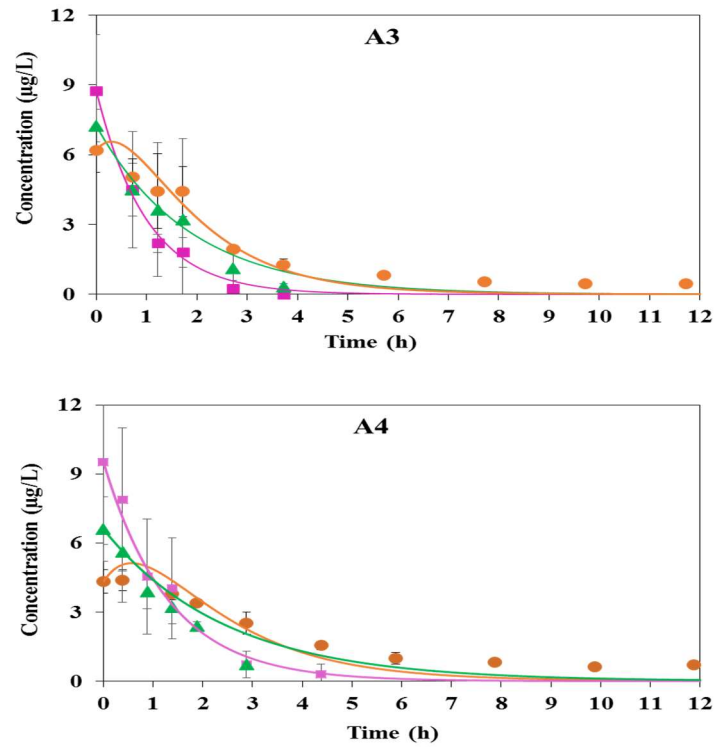
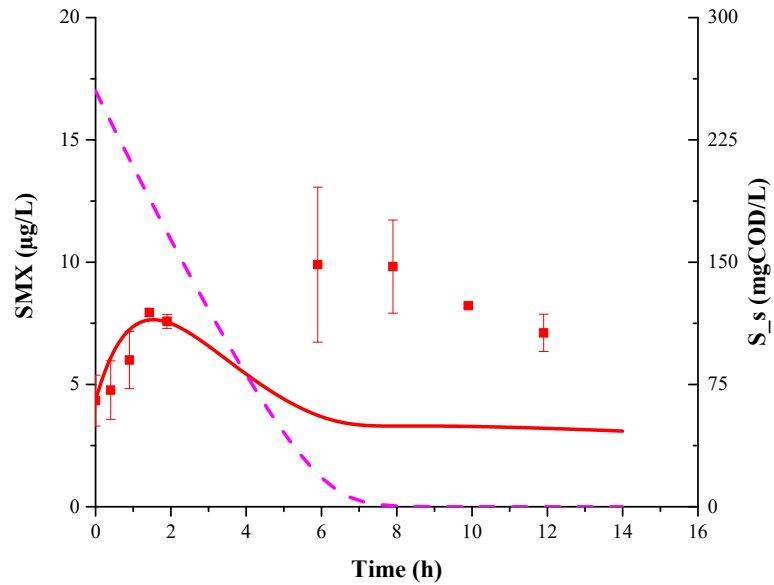


Fig. S5. Modelling results. Figure shows the results obtained for SMX and the two human metabolites, e.g. Ac-SMX and SMX-Glu, biotransformation in the anoxic batch tests (An1): non-bioaugmented activated sludge; (An2): bioaugmented activated sludge with *A. denitrificans* PR1. Full symbols represent measured concentrations plotted versus simulated concentrations during batch experiments. Dashed lines are the 95% confidence limits for the predicted concentrations.

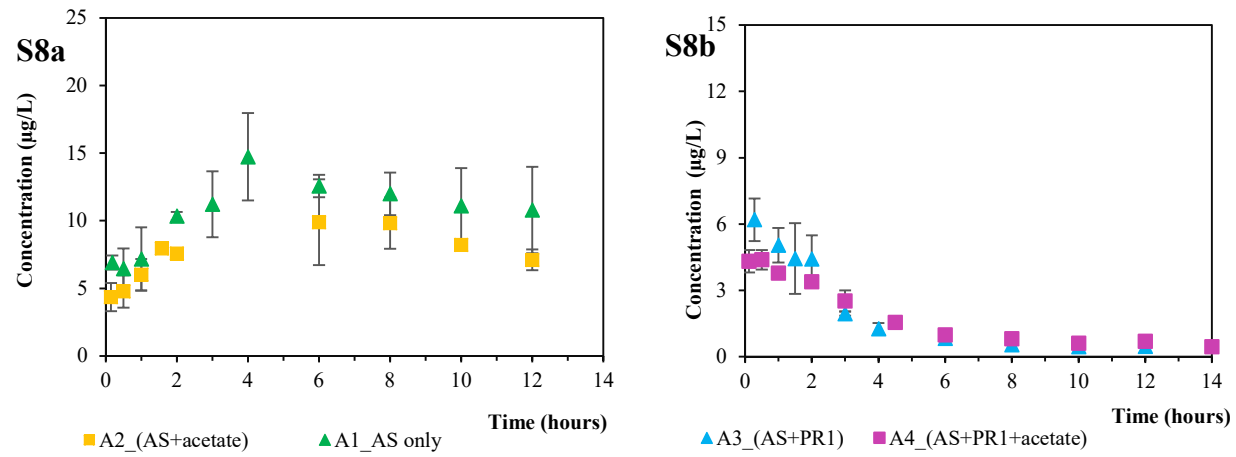




**Fig. S6.** Illustration of measured concentrations of SMX, Ac-SMX, and SMX-Glu (markers) and simulated (lines) as a function of time for aerobic batch tests (A1): non-bioaugmented activated sludge test; (A2): non-bioaugmented activated sludge test with supplementation of acetate as additional C-source; (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



**Fig. S7. Illustration of measured (markers) 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<sub>s</sub>) in the cometabolic model to enhance the SMX biotransformation by AS**



**Fig. S8. Measured concentrations of SMX as a function of time for the batch tests of non-bioaugmented AS (S8a) and bio-augmented one (S8b), with and without the addition of acetate under aerobic conditions. Error bars indicate the standard deviations for duplicates.**

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