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1 **Environmental risk assessment of antibiotics in agroecosystems: ecotoxicological effects on aquatic**
2 **microbial communities and dissemination of antimicrobial resistances and antibiotic**
3 **biodegradation potential along the soil-water continuum**

4

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21

22 **Abstract**

23 Antibiotics, have a wide application range in human and veterinary medicines. Being designed for
24 pharmacological stability, most antibiotics are recalcitrant to biodegradation after ingestion and can
25 be persistent in the environment. Antibiotic residues have been detected as contaminants in various
26 environmental compartments where they cause human and environmental threats, notably with
27 respect to the potential emergence and proliferation of antibiotic resistant bacteria. An important
28 component of managing environmental risk caused by antibiotics is to understand exposure of soil
29 and water resources to their residues. One challenge is to gain knowledge on the fate of antibiotics in
30 the ecosystem along the soil::water continuum, and on the collateral impact of antibiotics on
31 environmental microorganisms responsible for crucially important ecosystem functions. In this
32 context, the ANTIBIOTOX project aims at studying the environmental fate and impact of two
33 antibiotics of the sulfonamides class of antibiotics, sulfamethazine (SMZ) and sulfamethoxazole
34 (SMX).

35

36 **Keywords:** antibiotics; resistance gene; bacteria; biodegradation

37

38 **1. Environmental risks of antibiotics in agroecosystems**

39 Antibiotics (antimicrobials used to kill bacteria) are widely used in human medicine and in many
40 terrestrial and aquatic food animal production systems. Numerous antibiotics are recalcitrant to
41 degradation following ingestion and are excreted intact. Therefore, some antibiotics are widely
42 detected in terrestrial and aquatic matrices (Bottoni and Caroli 2015). In agroecosystems, antibiotic
43 residues can be transported from livestock production areas or from agricultural soils amended with
44 antibiotic-contaminated manure by surface runoff or infiltration, contaminating adjacent surface-
45 and groundwater resources (Boxall 2004). Once released in the environment, antibiotics may exert a
46 selection pressure favoring the emergence of bacteria harboring antimicrobial resistances (AMR)
47 even at low environmentally relevant concentrations (Gulberg et al. 2011). Indeed, the development
48 of antibiotic-resistant bacteria near confined animal feed operations (Chee-Sanford et al. 2001; Koike
49 et al. 2007; Sapkota et al. 2007), in soils treated with animal manures or in waste water treatment
50 plants (Gao et al. 2012) suggests that exposure to antibiotics can lead to the increase in abundance
51 of resistance genes in the environment (Heuer et al. 2011; Storteboom et al. 2010).

52 The World Health Organization has identified the increasing occurrence of AMR in human pathogens
53 as one of the most pressing contemporary global public health challenges. Due to the risks for
54 subsequent transfers of resistance factors to human pathogens an increasing reservoir of AMR
55 bacteria in the environment is of human health concern (Ashbolt et al. 2013; Bengtsson-Palme et al.,
56 2018). Promoting the 'One Health' concept, the United Nations and the European Commission
57 highlighted the need to develop approaches to better assess the risk for resistance selection in
58 natural microbial communities (Bengtsson-Palme and Larsson, 2016). In June 2017, the European
59 Commission adopted the new EU One Health Action Plan against AMR and the French Ministry of
60 Agriculture launched the new EcoAntibio national plan (2017-2021) with the objective of mitigating
61 the risk of AMR development. Managing the environmental risk of AMR is predicated on
62 understanding the exposure of soil and water resources to antibiotic residues, antibiotic-resistant
63 bacteria and antibiotic resistance genes (Blanco et al. 2009; Knapp et al. 2010; Pruden et al. 2013).
64 There is thus a need to identify environmental conditions that govern the fate of antibiotics in
65 agroecosystems including transfers along the soil-water continuum, as well as dissipation (e.g.
66 through biodegradation) and persistence (e.g. through retention due to sorptive processes) in the
67 different environmental compartments (Larsson et al. 2018). Although up to now, the major
68 concern about antibiotics is associated with the development and dissemination of resistance
69 mechanisms by bacteria (Heuer et al. 2011) and the implications of this process for both human and
70 animal health. Their sustained release to different environmental compartments and their bioactive
71 properties also raise serious issues about their impact on non-target organisms (Gonzales-Pleiter et
72 al. 2013). Briefly, and as recently underlined by Barra-Caracciolo et al. (2015) and Brandt et al. (2015),

73 much more knowledge concerning environmental emissions of antibiotics, their persistence,
74 degradation pathways and impacts on ecosystem health is required. Environmental risk assessments
75 should take into account terrestrial and aquatic microbial communities that undertake the main
76 ecosystem functions and services including the biodegradation of contaminants (Ghiglione et al.
77 2016).

78

79 **2. The case of Sulfonamides**

80 Among the antibiotics, the sulfonamides (SMs), one the oldest family on the market, are often
81 detected in aquatic and terrestrial matrices (Baran et al. 2006; Manzetti and Ghisi 2014; Deng et al.,
82 2018) because of the large volume used, high excretion rates, and relative persistence in soil (Brandt
83 et al. 2009). In a pioneering study, Kolpin et al. (2002) reported the detection of SMs in 80% of the
84 139 streams and rivers sampled in 1999 and 2000 across 30 states in the US. Since then SMs are
85 frequently detected in the environment (Hruska and Franek 2012) including rural streams and
86 connected rivers surrounding livestock production (Kolpin et al. 2002; Veach and Bernot 2011;
87 Bernot et al. 2013; Iglesias et al. 2014). It is noteworthy that sulfamethoxazole (SMX; used primarily
88 in human medicine) and sulfamethazine (SMZ; used primarily in food animal production) are among
89 the most often SM residues detected in various contaminations sources (waste water, digestate and
90 manure) and in the different compartments of the environment (soil, sediment, surface- and ground-
91 water) (Table 1). The repeated fertilization of cropland with animal manure containing veterinary
92 antibiotics (VAs) is one of the main contamination routes of surface water resources (Kemper 2008).
93 While their concentrations in surface water worldwide vary between a few and several dozen ng.L^{-1}
94 (Kolpin et al. 2002; Veach and Bernot 2011; Bernot et al. 2013; Iglesias et al. 2014; Matongo et al.
95 2015; Grenni et al. 2018), peak concentrations can sometimes reach several $\mu\text{g.L}^{-1}$ in different rivers
96 (Yargau et al. 2007; Peng et al. 2008; Lacina et al. 2012; Matongo et al. 2015). In France, a survey
97 performed in the Seine River revealed that SMX was detected in all samples, with a maximum
98 concentration of 544 ng.L^{-1} (Tamtam et al. 2008). In the US, a study showed that 20% of groundwater
99 samples were contaminated with SMX at concentrations of up to $1.0 \mu\text{g.L}^{-1}$ which are in the range of
100 pesticide concentrations observed in agricultural streams (Barnes et al. 2008). A recent review of the
101 maximum concentrations of the most frequently detected SMs in contamination sources and in
102 different environmental compartments including soils, surface and ground waters can be found in
103 Deng et al. (2018).

104 SMs are broad-spectrum bacteriostatic agents that inhibit the biosynthesis of folic acid through
105 competitive inhibition of dihydropteroate synthase, blocking the condensation of 6-hydroxymethyl-
106 7,8-dihydropteridine pyrophosphate to para-aminobenzoic acid (Petri 2001). Although known as
107 recalcitrant compounds, SMs can be metabolized, the most frequently detected metabolites being

108 the 3-amino-5-methylisoxazole and 2-amino-4,6-dimethylpyrimidine for SMX and SMZ, respectively
109 (Ricken et al. 2013) (Figure 1). Strains belonging to the genus *Rhodococcus*, known to co-metabolize
110 hydrocarbons, co-metabolically degrade SMX as well as other SMs such as sulfamethizole (Gauthier
111 et al. 2010; Larcher and Yargeau 2011). Other strains belonging to the class of γ -proteobacteria have
112 been shown to co-metabolically degrade various SMs (Zhang et al. 2012a; Zhang et al. 2012b; Mao et
113 al. 2018). The co-metabolic transformation or biodegradation of sulfonamide antibiotics by various
114 microorganisms has recently been reviewed (Deng et al. 2018; Chen and Xie 2018; Wang and Wang
115 2018).

116 Chronic or punctual exposure of natural soil microbial communities to SMs can enhance their
117 capacities to degrade this class of antibiotic. Indeed, repeated long-term treatment of agricultural
118 soil with SMZ resulted in an increase of SMZ mineralization capacities of autochthonous microbial
119 communities, at least partially due to the selection of a resistant bacterial population
120 (*Microbacterium* sp. strain C448) able to metabolize and grow at the expense of SMZ and other
121 sulfonamides (Topp et al. 2013). *Microbacterium* C448 mineralizes the benzylic ring of the molecule,
122 excreting the pyrimidine moiety as an end product of metabolism (Topp et al. 2013) (Figure1). Two
123 other *Microbacterium* strains, BR1 (Ricken et al. 2013) and SDZm4 (Tappe et al. 2013) were isolated
124 from a waste water treatment plant and from a lysimeter exposed to pig manure, respectively.
125 Biodegradation of SMs was subsequently reported in members of the phyla Proteobacteria
126 (*Pseudomonas* (Jiang et al. 2014), *Ochrobactrum* (Mulla et al. 2018) and *Acinetobacter* (Wang et al.
127 2018)) and Actinobacteria (*Microbacterium* (Ricken et al. 2013; Tappe et al. 2013; Topp et al. 2013);
128 *Arthrobacter* (Deng et al. 2016), *Gordonia* (Mulla et al. 2018), and *Leucobacter* (Reis et al. 2018)). SM
129 degradation of *Microbacterium* sp. BR1 is initiated by an *ipso*-substitution (Ricken et al. 2013)
130 followed by a ring cleavage in the downstream pathway (Ricken et al. 2015). The *sadABC* gene cluster
131 encodes two monooxygenases (SadA and SadB) and one flavine mononucleotide (FMN) reductase
132 (SadC). SadA and SadC initiate the transformation of SMs yielding to the release of 4-aminophenol.
133 This intermediate is then transformed by SadB and SadC to 1,2,4-trihydroxybenzene which is entirely
134 mineralized (Ricken et al. 2017). This gene cluster is nearly identical (between 97 and 99% similarity)
135 in the draft genomes of *Microbacterium* sp. C448 (Martin-Laurent et al. 2015), *Microbacterium* sp.
136 SDZm4 (Tappe et al. 2013) and *Arthrobacter* sp. D2 (Deng et al. 2016), suggesting that it might be
137 dispersed through horizontal exchange in the *Actinobacteria* phylum (Ricken et al. 2017). Overall, the
138 widespread distribution of sulfonamide-biodegrading bacteria presumably decreases the
139 environmental persistence of these antibiotics, and thus the selection pressure for resistance
140 development. Bioaugmentation of soil with *Microbacterium* sp. strain C448 decreased the
141 persistence of SMZ, indicating that bioremediation is an option for dealing with pollution hotspots
142 (Hirth et al. 2016).

143

144 In addition to their crucial role in the dissipation of pollutants including SMs, natural microbial
145 communities in soil and water are responsible for a range of ecosystem functions and services.
146 However, ecotoxicological effects of antibiotics and notably of SMs on microbial communities are still
147 understudied, and there is a crucial need to investigate their impact on biogeochemical processes to
148 further assess their possible adverse effects on ecosystem functioning (Roose-Amsaleg and Laverman
149 2016). This is notably the case for surface waters such as small rivers draining agricultural areas
150 including livestock production farms or crop fields amended with manure. In these lotic ecosystems,
151 most of the microbial activities are assumed by benthic communities which develop on hard and soft
152 immersed substrates and which are composed of both phototrophic (mainly algae and
153 cyanobacteria) and heterotrophic (mainly bacteria, fungi, and protozoa) microorganisms.
154 Pharmaceutical accumulation in these microbial assemblages has been occasionally reported (Writer
155 et al. 2013; Wunder et al. 2016; Huerta et al. 2016). For instance, SMX tends to progressively
156 accumulate in river biofilms (Corcoll et al. 2015). Exposure of aquatic microbial communities to
157 antibiotics spiked at chronic concentration in microcosm was also found to induce resistance
158 strategies at community level including microbial degradation of antibiotics (Vila-Costa et al., 2017).
159 Environmental exposure to antibiotics potentiates the environmental reservoir of antibiotic
160 resistance increasing the threat to both human and animal health (Wright 2010; Ashbolt et al. 2013;
161 Finley et al. 2013; Berendonk et al. 2015). One can distinguish two kinds of resistance to
162 sulfonamides. On the one hand, 3- of 6-bp duplication within *sulA*, the chromosomal gene encoding
163 for dihydropteroate synthase, was shown to confer resistance to sulfonamides to *Streptococcus*
164 *pneumoniae* (Lopez *et al.*, 1987; Maskell *et al.* 1997). On the other hand, sulfonamide resistance
165 involves the acquisition of an additional dihydropteroate synthase (i.e. *sul1*, *sul2* and *sul3*) on a
166 mobile genetic element that is not inhibited by this class of antibiotics and that bypass the inhibition
167 of the enzyme encoded by the chromosomal genes. The *sul* resistance genes, located within
168 integrons (Type 1 integrase) and carried on conjugative plasmids, are widely distributed in SM-
169 resistant bacteria (Heuer et al. 2011). These genes have been detected in various aquatic ecosystems
170 (e.g. Pei et al. 2006; Pruden et al. 2007; Luo et al. 2010; McKinney et al. 2010; Stoll et al. 2012). In
171 addition, zinc was found to increase the abundance of *sul* resistance genes in intestine of weaned
172 pigs fed with high dietary zinc supplementation as an alternative to antibiotics (Vahjen et al. 2015).
173 This suggests that heavy metals can co-select antibiotic resistances by exerting a selection pressure
174 favorable to the maintenance of mobile mosaic gene structures harboring both heavy metal and
175 antibiotic resistances in the environment (Vahjen et al. 2015). Nevertheless, there are relatively few
176 studies documenting their dispersal and survival from different sources of contaminations in
177 agroecosystems microbiota, particularly with respect to the terrestrial to aquatic continuum.

178 Ecotoxicological risks and effects of SMs in agroecosystems are also largely understudied. Recently, a
179 few papers proposed different types of usage pattern-based exposure to prioritize antibiotics to be
180 considered in environmental risk assessment (ERA) (Di Nica et al. 2015; Menz et al. 2015). These
181 approaches are based on the calculation of the risk quotient (RQ) as the ratio of predicted
182 environmental concentration (PEC) over the predicted no effect concentration (PNEC). RQ values
183 permit to predict the environmental risk for each antibiotic in the phase II of environmental risk
184 assessment. However, besides *a priori* ERA of SMs including toxicological test on very sensitive
185 species such as *Anabaena*, very few studies report their ecotoxicological impact on microbial
186 communities. The first studies dealing with the effects of SMs on aquatic microbial communities
187 focused on bacteria from activated sludge (Xia et al. 2012; Collado et al. 2013). SMX was found to
188 shift bacterial community structure but did not modify microbial functions such as organic carbon
189 and nitrogen removal. However, SMs induced large shifts in the transcriptome of microbial
190 communities in the gene categories related to N, P and C cycling (Yergeau et al. 2012). A negative
191 exponential relationship between denitrifying gene abundance and SMZ concentrations was
192 observed in estuarine and coastal sediments (Hou et al. 2015). This decrease in the presence of SMZ
193 led to a reduction of denitrification rates. A mechanistic model to interpret the toxic effects of SMs
194 on nitrification in activated sludge system was proposed (Huang et al. 2016). Effects of SM exposure
195 on fungi and algae co-existing with bacteria in aquatic biofilms are still unknown. In soil, microbial
196 communities exposed to SMs experienced an overall reduction in the bacteria/fungi biomass ratio
197 suggesting that fungi consumed nutrients released by bacteria killed by the antibiotic (Gutierrez et al.
198 2010; Pinna et al. 2012). This response might also occur to microbial communities in antibiotic-
199 contaminated river ecosystems but may be modulated by the difference in proportion of bacteria,
200 algae and fungi among different benthic substrata (cobbles, sediment, leaves) (Artigas et al. 2009;
201 Mora-Gomez et al. 2016). Simple additive effects occur with binary mixtures of SMs (SMZ and SMX)
202 on the growth inhibition of the freshwater green algae *P. subcapitata* (Yang et al. 2008). Diatoms and
203 cyanobacteria were found to be more sensitive to pharmaceutical mixtures including SMs than to
204 single antibiotics (Van der Grinten et al. 2010; Perron and Juneau 2011). Few studies have assessed
205 how changes in microbial composition and activity of river biofilms in response to antibiotic exposure
206 could lead to perturbation of ecosystem functions like nutrient cycling.

207

208 **3. The ANTIBIOTOX project**

209 Within this context, ANTIBIOTOX ('Fate of antibiotics and associated resistance genes in
210 agroecosystems: ecotoxicological risk for functional microbial communities of receiving river
211 systems) is an innovative project funded by the French ANR (Agence National de Recherche; contract
212 n° ANR-17-CE34-0003, 2018-2022) which aims to develop a multidisciplinary approach combining

213 chemistry, biochemistry, environmental microbiology, bacterial genetics, molecular biology and
214 ecotoxicology to (i) study transcriptomic and proteomic regulation during the time-course of SMs
215 biodegradation in *Microbacterium* sp. C448, (ii) characterize the biodegradation pathways and
216 estimate the ecotoxicological impact of microbial biodegradation of SMs in aquatic and terrestrial
217 compartments of agroecosystems, (iii) monitor the dissemination of SM-resistant and SM-degrading
218 bacteria and genes from agricultural soil to adjacent water resources, (iv) estimate the impact of SM
219 residues (and transformation products) on the abundance of resistant and degrading microbes and
220 microbial functions in the aquatic ecosystem and (v) communicate and promote results as well as
221 contribute to higher education programs in order to disseminate the main outcomes of this research
222 program. ANTIBIOTOX is focused on two SMs, namely sulfamethoxazole and sulfamethazine which
223 are used in human medicine and in food animal production. For example, 20% of the veterinary
224 antibiotics (147 tons of active substance per year) sold in France are in the SM class (ANSES 2015).
225 Sulfonamides are frequently detected in various aquatic environments (Deng et al., 2018). Overall,
226 this multidisciplinary project is expected to provide new insights in the ecotoxicological impact of
227 veterinary/human antibiotic residues on functional microbial diversity along the soil-water
228 continuum.

229

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236

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490

491

492 **Table 1.** Occurrence of sulfamethoxazole and of sulfamethazine in different environmental matrices
 493 (soil, sediment, surface- and ground-water) and in various sources of contamination (waste water,
 494 digestate and manure)

Antibiotic	Matrice	Place ^a (#of studies)	Concentrations ^{b,c}	Reference	
Sulfamethoxazole	Soil	Ch (4), Sp (1), US (1)	19.3 ^b	De Vries and Zhang, 2016	
	Sediment	Ch (5), US (2), K (1), Sp (1)	0.5 ^b	De Vries and Zhang, 2016	
		Surface water	It	1.8-2.4 ^c	Grenni et al., 2018
			It	68 ^c	Grenni et al., 2018
			Ca	578 ^c	Yargau et al., 2007
	US	150 ^c	Kolpin et al., 2002		
	Ground water	Nt(3)	50-75 ^c	Chitescu et al., 2012	
	Waste water	US (7)	98-2200 ^c	Batt et al., 2008	
	Digestate	Ge	880 ^b	Ratsak et al., 2013	
Manure	Ge	nd	Ratsak et al., 2013		
Sulfamethazine	Soil	Ch (4), US (1), K (1)	3.2 ^b	De Vries and Zhang, 2016	
	Sediment	Ch (6), US (2), K (1)	2.9 ^b	De Vries and Zhang, 2016	
	Surface water	US	6 ^c	Bernot et al., 2013	
		US	20 ^c	Kolpin et al., 2002	
	Ground water	Nt (2)	21 ^c	de Weert and Smedes, 2015	
	Waste water	Ch	382 ^c	Chunhui et al., 2016	
	Digestate	Ge	160 ^b	Ratsak et al., 2013	
	Manure	Ge	700 ^b	Ratsak et al., 2013	

495 ^aChina (Ch), Germany (Ge), Korea (K), Netherland (Nt), Spain (Sp), United States (US); ^bin $\mu\text{g.kg}^{-1}$; ^cin ng.L^{-1}

496

497 **Figure Caption**

498 **Figure 1.** Overview of the main microbial biodegradation pathways of SMX and SMZ (co-metabolic
499 pathways: brown arrows; metabolic pathways: black arrows). 1: 3-amino-5-methylisoxazole, 2: 4-
500 aminobenzenesulfonamide, 3:4-aminothiophenol, 4: aniline, 5: 4-aminophenol, 6: 2,4-
501 dihydroxyphenol, 7: hydroquinone, 8: N-hydroxymethyl-SMX, 9: N-hydroxyacetyl-SMX, 10: 4-
502 hydroxybenzenesulfonic acid, 11: 3-hydroxylamine-amino-5-carboxyl, 12: 4-hydroxy-N-(5-methyl-3-
503 isoxazole)benzene-1-sulfonamide, 13: 4-aminobenzenesulfonic acid. A: 2-amino-4,6-
504 dimethylpyrimidine.

