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

Fabrice Martin-Laurent<sup>1</sup>, Edward Topp<sup>2</sup>, Loren Billet<sup>1</sup>, Isabelle Batisson<sup>3</sup>, Cédric Malandain<sup>4</sup>, Pascale Besse-Hoggan<sup>5</sup>, Soizic Morin<sup>6</sup>, Joan Artigas<sup>3</sup>, Chloé Bonnineau<sup>7</sup>, Laura Kergoat<sup>7</sup>, Marion Devers-Lamrani<sup>1</sup> and Stéphane Pesce<sup>7</sup>

<sup>1</sup>AgroSup Dijon, INRA, Univ. Bourgogne, Univ. Bourgogne Franche-Comté, Agroécologie, F-21000 Dijon, France

<sup>2</sup>Agriculture and Agri-Food Canada, University of Western Ontario, London, ON N5V 4T3, Canada

<sup>3</sup> Université Clermont Auvergne, CNRS, Laboratoire Microorganismes : Génome et Environnement, F-63000 Clermont–Ferrand, France

<sup>4</sup> ENOVEO, F-69000 Lyon, France

<sup>5</sup> Université Clermont Auvergne, CNRS, Sigma Clermont, Institut de Chimie de Clermont-Ferrand, F-63000 Clermont–Ferrand, France

<sup>6</sup>Irstea, UR EABX, F-33610 Cestas, France

<sup>7</sup>Irstea, UR RiverLy, F-69100 Lyon-Villeurbanne, France

**Correspondence:** Fabrice Martin-Laurent (fabrice.martin@inra.fr)

## **Abstract**

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

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

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

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

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

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

## **2. The case of Sulfonamides**

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

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

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

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

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

### 3. The ANTIBIOTOX project

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



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

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491

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

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

<sup>a</sup>China (Ch), Germany (Ge), Korea (K), Netherland (Nt), Spain (Sp), United States (US); <sup>b</sup>in  $\mu\text{g.kg}^{-1}$ ; <sup>c</sup>in  $\text{ng.L}^{-1}$

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.

