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## Sulfamethoxazole biodegradation and impacts on soil microbial communities in a Bolivian arid high altitude catchment.

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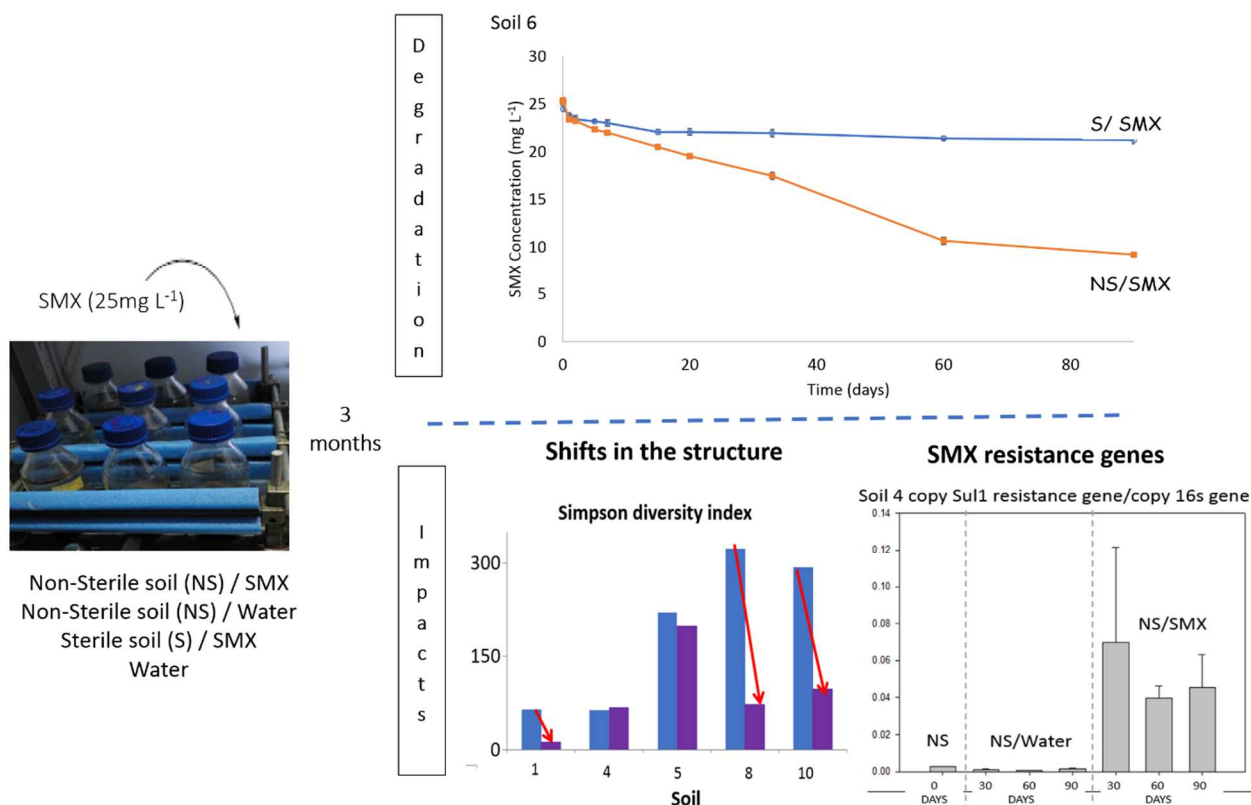
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Graphical abstract:



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19 **ABSTRACT:**

20 The processes controlling antibiotics fate in ecosystems are poorly understood, yet their presence  
21 can inhibit bacterial growth and induce the development of bacterial resistance. Sulfamethoxazole  
22 (SMX) is one of the most frequently detected sulfonamides in natural environments due to its low  
23 metabolism and molecular properties. This work presents pioneering results on SMX  
24 biodegradation and impact in high altitude soils (Bolivian Altiplano), allowing a better  
25 understanding of the persistence, spread and impact of this antibiotic at the global watershed scale.  
26 Our results showed significant dissipation of SMX in relation to its adsorption, hydrolysis and  
27 biotransformation. However, biodegradation appears to be lower in these mountain soils than in  
28 lowland soils as widely described in the literature. The half-life of SMX ranges from 12 to 346 days  
29 in non-sterile soils. In one soil, no biotic degradation was observed, indicating a likely high  
30 persistence. Biodegradation was related to OC content and to proximity to urban activities.  
31 Regarding the study of the impacts of SMX, the DGGE results were less sensitive than the  
32 sequencing. In general, SMX strongly changes the structure and composition of the studied soils at  
33 high altitudes, which is comparable to the observations of other authors in lowland soils. The  
34 phylum Actinobacter showed high sensitivity to SMX. In contrast, the abundance of  $\gamma$ -  
35 proteobacteria remained almost unchanged. Soil contamination with SMX did not lead to the  
36 development of the studied resistance genes (*sul1* and *sul2*) in soils where they were absent at the  
37 beginning of the experiment. Thus, the presence of SMX resistance genes seems to be related to  
38 irrigation with wastewater carrying the studied resistance genes.

39

## 40 **Introduction**

41 Over the last few decades, the consumption of antibiotics has continuously increased in all  
42 countries. In a recent study <sup>1</sup> showed that antibiotic consumption increased by up to 65% in all  
43 countries between 2000 and 2015, particularly in low- and middle-income countries (LMICs). As a  
44 result, these so-called emerging contaminants are increasingly detected in wastewater and natural  
45 ecosystems worldwide <sup>2-4</sup>. The ecotoxicological impact of antibiotics is fairly well known <sup>5,6</sup>. It was  
46 showed that in the soils of the Katari watershed, sulfamethoxazole (SMX) at concentrations close to  
47 environmental levels significantly induce the accumulation of nitrogen oxide (NO) and NO-derived  
48 reactive species and the formation of 4-nitro-SMX and 4-nitroso-SMX<sup>7</sup>. Furthermore, SMX  
49 resistance genes *sul1* and *sul2* were often detected in the basin <sup>8</sup>.

50 Although human intake of pharmaceuticals through vegetables consumption does not seem to pose  
51 a threat to human health <sup>9</sup>, pharmaceuticals and their metabolites have been shown to alter cell  
52 functions and gene expression (light sensitivity, allergic reactions, malformations), or to act as  
53 endocrine disrupting compounds (EDCs) and induce resistances in microorganisms. Bacterial  
54 resistance to antibiotics has now become a central issue as it can lead to global health problems for  
55 animals and humans <sup>10,11</sup>. On the other hand, the main drivers controlling the environmental fate of  
56 pharmaceuticals such as antibiotics are poorly documented due to the complexity of the undergone  
57 processes (e.g. sorption, complexation, biotic or abiotic transformations) determined by the specific  
58 properties of both the environment (physico-chemical properties and climatic factors) and the  
59 contaminants <sup>12</sup>.

60 The natural attenuation of antibiotics in the environment can cushion the increasing spread of  
61 contamination. Unfortunately, little is known about the degradation of antibiotics under natural  
62 conditions, especially in high altitudinal arid zones where the effects of climate change could  
63 contradictory promote or reduce the risk associated with antibiotics, by the increased spreading of  
64 antibiotics (through increased rainfall and flooding) or their enhanced degradation (at higher  
65 temperatures).

66 SMX, which belongs to the sulfonamide antibiotic family, is one of the most frequently detected  
67 antibiotic in natural environments<sup>3,13-16</sup> primarily because they are poorly absorbed and metabolized  
68 in organisms<sup>11,17</sup>. Observed SMX concentrations ranging from 5 to about 15,000 ng L<sup>-1</sup> in the  
69 surface waters of the Katari watershed in the Bolivian Highlands, indicating a quite variable  
70 persistence in such environments<sup>8</sup>.

71 Overall sulfonamides bind strongly to soil components and form stable residues<sup>12</sup>. The detailed  
72 mechanisms of SMX degradation in activated sludge and in aquatic and terrestrial ecosystems have  
73 been examined in several studies. Authors<sup>18-21</sup> suggested that natural biodegradation may play an  
74 important role in the dissipation of SMX in aquatic or terrestrial environments and that degradation  
75 occurred at a lower rate under anaerobic conditions<sup>22</sup>. It was showed that SMX is eliminated via  
76 sorption and biodegradation in activated sludge<sup>20</sup>. Similarly, it was observed that bacterial  
77 degradation activity increases if SMX is previously photodegraded in water<sup>23</sup> and that SMX  
78 sorption dominates in soil during the first moments of soil-pollutant contact and then gives way to  
79 biodegradation<sup>24</sup>. It was observed that biodegradation was the main process for SMX removal  
80 compared to sorption by SMX acclimatized cultures<sup>25</sup> and that microbial interactions drive the  
81 SMX complete catabolism in activated sludge<sup>26</sup>. Hydrolysis is considered one of the most  
82 important pathways for abiotic degradation, but sulfonamides are known to be less susceptible to  
83 hydrolysis<sup>12</sup>.

84 The impact of antibiotics on the structure and functioning of bacterial communities in soil and water  
85 has also been widely studied<sup>10,27-29</sup> as well as their effect on the development and spreading of  
86 antibiotic resistance within bacteria<sup>29,30,30</sup>. They affect their enzyme activity and ability to  
87 metabolize different carbon sources, modify the microbial biomass, the relative abundance of  
88 different groups and the presence of many types of antibiotic resistance genes (ARGs)<sup>12,25</sup>.

89 In the case of sulfonamides it is known that bacterial resistance is mainly mediated by genes "*sul1*",  
90 "*sul2*" and "*sul3*"<sup>31,32</sup>, they have been found in large quantities in a wide range of environmental

91 matrices<sup>33,34</sup>, thus appearing as good candidates for monitoring bacterial resistance to sulfonamides  
92 in the environment.

93 Although the degradation of SMX and the impact of sulfonamides in soil and water have been  
94 widely studied, understanding of its fate remains poor at the watershed scale, especially with regard  
95 to the immense diversity and heterogeneity of soil biogeochemical properties at such a scale.  
96 Similarly, a large amount of data is available on the degradation of pharmaceuticals in soil on a  
97 small scale, but quantification and prediction of these processes at the watershed scale under natural  
98 conditions remains difficult, especially under extreme conditions such as those at high altitudes.

99 This study is the first of its kind on the natural soils of the Bolivian Altiplano. By studying 10 soils  
100 representative of the pedology of the Titicaca basin, we first sought to advance our knowledge of  
101 the biodegradation of sulfonamide-antibiotics in natural high-altitudinal arid soils at a catchment  
102 scale. The second goal was to document sulfonamide-antibiotics impact on the structure of soil  
103 bacterial communities of disturbed top layer (i.e. 0-10 cm below surface) and to study the  
104 development of sulfonamide resistance genes, using biomolecular techniques. SMX was used as a  
105 model sulfonamide antibiotic. The results were discussed in the light of field SMX concentration  
106 levels, main physico-chemical properties of soils and land use.

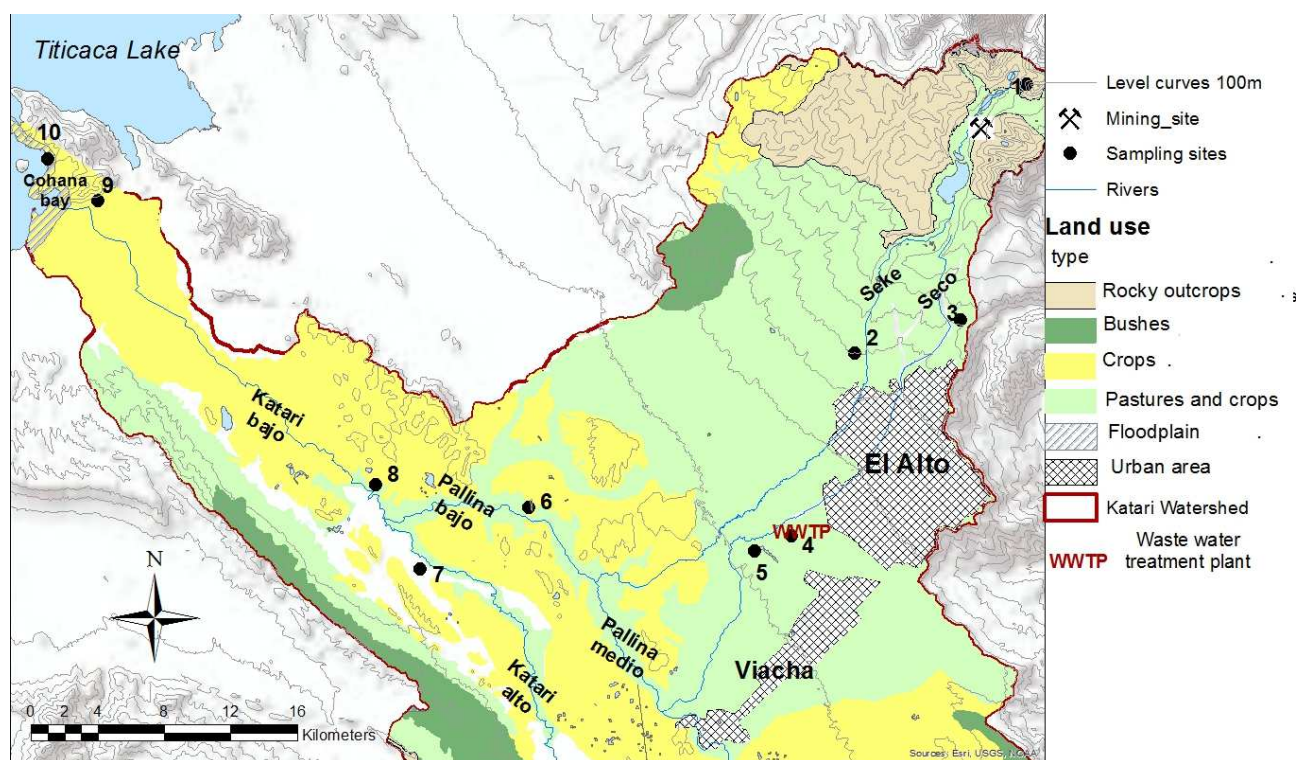
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## 108 **Materials and methods**

109 **Sampling, soil properties and SMX initial concentrations.** The 0-10cm layer of soil was  
110 collected at ten different locations in the Katari watershed (Figure 1) from 4700 m to 3830 m  
111 altitude. Natural sites (soil 1, 2 and 3), agricultural sites (soils 6, 7 and 8), urban sites (soils 4  
112 and 5) and along the edge of Lake Titicaca (soils 9 and 10). Collection sites were selected to  
113 represent best the places where anthropogenic activities occur. Soils 9 and 10 may be subject to  
114 flooding. Soils 1, 2, 3, 6 and 7 were collected close to the surface waters network (around 100 m  
115 away) but are not or very rarely flooded. Soils 4 and 5 were collected at the entrance (below the  
116 wastewater collector pipe) and inside the wastewater treatment plant respectively.

117 Samples were sieved at <2mm and transported in plastic bags to the IGE Laboratory (France) at  
118 4°C. Extensive soil description and use can be found in Archundia et al. (2019)<sup>35</sup>.

119 The main physico-chemical properties and soil type of the ten studied soils are summarized in  
120 supporting information (SI. 1). The SMX contents measured in these soils is presented in Archundia  
121 et al. (2017)<sup>8</sup>, initial concentrations ranged between <LD (0.1 ng L<sup>-1</sup>) and 18 µg kg<sup>-1</sup>. The  
122 correspondences of soils identification between this paper and Archundia et al. (2017)<sup>8</sup> are the  
123 following: 1 (R), 2 (Not analyzed), 3 (SI), 4 (I), 5 (O), 6 (U5), 7 (K1), 8 (K2), 9 (K3) and 10 (CB1).



124  
125 **Figure 1.** Study site and location of studied soils.

126  
127 **Experimental design.** The biodegradation experiments were conducted in triplicate within two  
128 months following soils sampling (stored at 4°C, close to the temperature at the time of sampling).  
129 The experiments were conducted in 250-ml glass bottles with soil slurry according to the  
130 procedure described by Martins and Mermoud (1998). Three different treatments were carried  
131 out in duplicate: i) non-sterile soils with sterile water (NS-C) used as blanks, ii) sterile soils with  
132 SMX (S-SMX) used to assess abiotic degradation and sorption of SMX, and iii) non-sterile soils

133 with SMX (NS-SMX). Additionally, an internal standard control tests with autoclaved soils and  
134 sterile water were performed systematically to account for the presence of matrix effects (linked to  
135 soil autoclaving) possibly affecting HPLC measurements. Controls showed the absence of a peak at  
136 the SMX retention time in solutions of non-SMX spiked autoclaved soils. Soil solutions were  
137 prepared with sterile HPLC-grade water. The initial concentration of SMX was set at  $\sim 25 \text{ mg L}^{-1}$   
138 (SMX water solubility) by first dissolution in methanol and application of 0.1mL of SMX in soil  
139 suspensions. This intermediate antibiotic concentration is more likely to induce contrasting  
140 degradation rate constants among the studied soils, compared to very low concentrations<sup>36</sup>, allowing  
141 the determination of relative biodegradation constants and to study the worst case scenario. The  
142 ionic strength of the soil solution was fixed at a constant level by adding a  $\text{CaCl}_2$  solution with an  
143 equilibration time of 12h.

144 In addition, batches containing 200 ml of SMX solution (duplicate, same ionic strength and SMX  
145 concentration) were incubated under the same conditions to account for the hydrolysis and to  
146 exclude any possible effect of its adsorption on the glass. Sterile soils were obtained by two  
147 consecutive autoclaving ( $121^\circ\text{C}$  and 1 bar for 1.5 h). Sterility was verified by bacterial plate counts  
148 of soil aliquots using Luria-Bertani agar plates.

149 The most appropriate soil/solution ratio was tested (preliminary tests, data not shown) and set at  
150 1:10. Soil batches were therefore prepared by placing 20g of air dried soil in glass flasks closed  
151 with cotton rubbers, equilibrated with 200 ml of  $\text{CaCl}_2$  solution ( $1.1 \text{ g L}^{-1}$ ) and spiked with  
152 appropriate amounts of SMX. SMX (98% purity) was purchased from LKT Laboratories, Inc. The  
153 batches were incubated for 3 months under stirring at 350 rpm in the dark at constant  
154 temperature ( $20^\circ\text{C} \pm 2$ ). At 0, 1, 2, 5, 7, 15, 20, 33, 60 and 90 days, 1.5ml of soil slurry was  
155 collected in all batches and centrifuged to separate solid and liquid<sup>36</sup>. The supernatant of each  
156 sample was stored at  $-20^\circ\text{C}$  until analysis. SMX concentrations were determined by HPLC – UV  
157 (SI. 2).



158

159 **SMX impact on soil bacterial communities.** Impacts were studied in soils 1, 4, 5, 8 and 10,  
160 representing all pedological conditions in the basin, using Denaturing Gradient Gel Electrophoresis  
161 (DGGE), high-throughput sequencing and qPCR techniques. Solely soil microbial communities of  
162 disturbed top layer (i.e. 0-10 cm below surface) soil samples were studied. Duplicate soil samples  
163 were collected in the NS-SMX soils and the NS-C Control soils, at 1, 7, 30, 60 and 90 days. To  
164 evaluate the SMX impact these samples were compared to samples of untreated soils (T0). DNA  
165 was extracted from the soil samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals)  
166 following the manufacturer's protocol. Raw DNA was analyzed by agarose gel electrophoresis (1%)  
167 compared to a range of calf thymus DNA (Biorad France) and UV visualization of ethidium  
168 bromide stained gels with a Gel Doc™ XR Molecular imager device (Biorad). The detailed  
169 procedure used to perform the *16S rDNA* amplification for DGGE analysis and the DGGE analysis  
170 itself can be found on SI. 3.

171 Similarity dendrograms (clustering based on the Pearson correlation coefficient) by the  
172 Agglomerative hierarchical clustering (AHC) method were performed on the basis of a binary  
173 matrix representing the presence or absence of a band in the DGGE analysis by using the XLSTAT  
174 2014.4.10 software with samples obtained 1, 7 and 30 days after the SMX contamination.

175 The similarities were also studied by comparing different parameters calculated from the DGGE  
176 profiles measured 0 and 30 days after SMX spiking: diversity index (Shannon, Simpson's) and  
177 Richness. The details regarding the estimation of these parameters are found in SI. 4.

178 Based on the DGGE results, only T0 and T30 samples of all conditions were analyzed by high-  
179 throughput sequencing in a MiSeq System to assess the SMX impact at the microbial community  
180 level, i.e. the changes in the structure of bacterial communities. The detailed procedure can be  
181 found on SI. 5.

182 qPCR assays were used to determine the prevalence of SMX resistance genes (RGs) in the  
183 bacterial communities of the studied soils, following the procedure described by Heuer et al. (2008)  
184 and Heuer and Smalla (2007)<sup>37,38</sup>. The targeted ARG's were *sul1* and *sul2*. qPCR was performed at  
185 30, 60 and 90 days after SMX soil contamination in triplicate. The real-time PCR primer sets used  
186 are listed SI. 6. The details of the qPCR procedure and the generation of the plasmids carrying the  
187 target genes for calibration curves are found on SI. 7. The qPCR efficiencies ranged within 90 and  
188 102% and the R<sup>2</sup> values were between 0.98 and 0.99 for all calibration curves.

189 Sample quality control was performed for DNA extraction and all biomolecular analysis by  
190 including negative control samples (in triplicate).

191 **Statistical analysis.** The statistical significance of differences in structural parameters and diversity  
192 indices (calculated from DGGE fingerprints and high-throughput sequencing) between treatments  
193 was tested by analysis of variance (ANOVA) and differences between the calculated means were  
194 tested by paired multiple comparisons with the Bonferroni correction (n=2). Differences in  
195 taxonomic composition of microbial communities of the disturbed soil top layer (0-10 cm) were  
196 also studied. Statistical significance was determined at the confidence level of 0.05. Environmental  
197 and microbial variables were compared statistically by applying the Pearson's correlation  
198 coefficient. All tests were performed using the statistical software R 3.1.1. The significance level  
199 was set at 0.05 for all statistical analyses.

200

## 201 **Results and discussion**

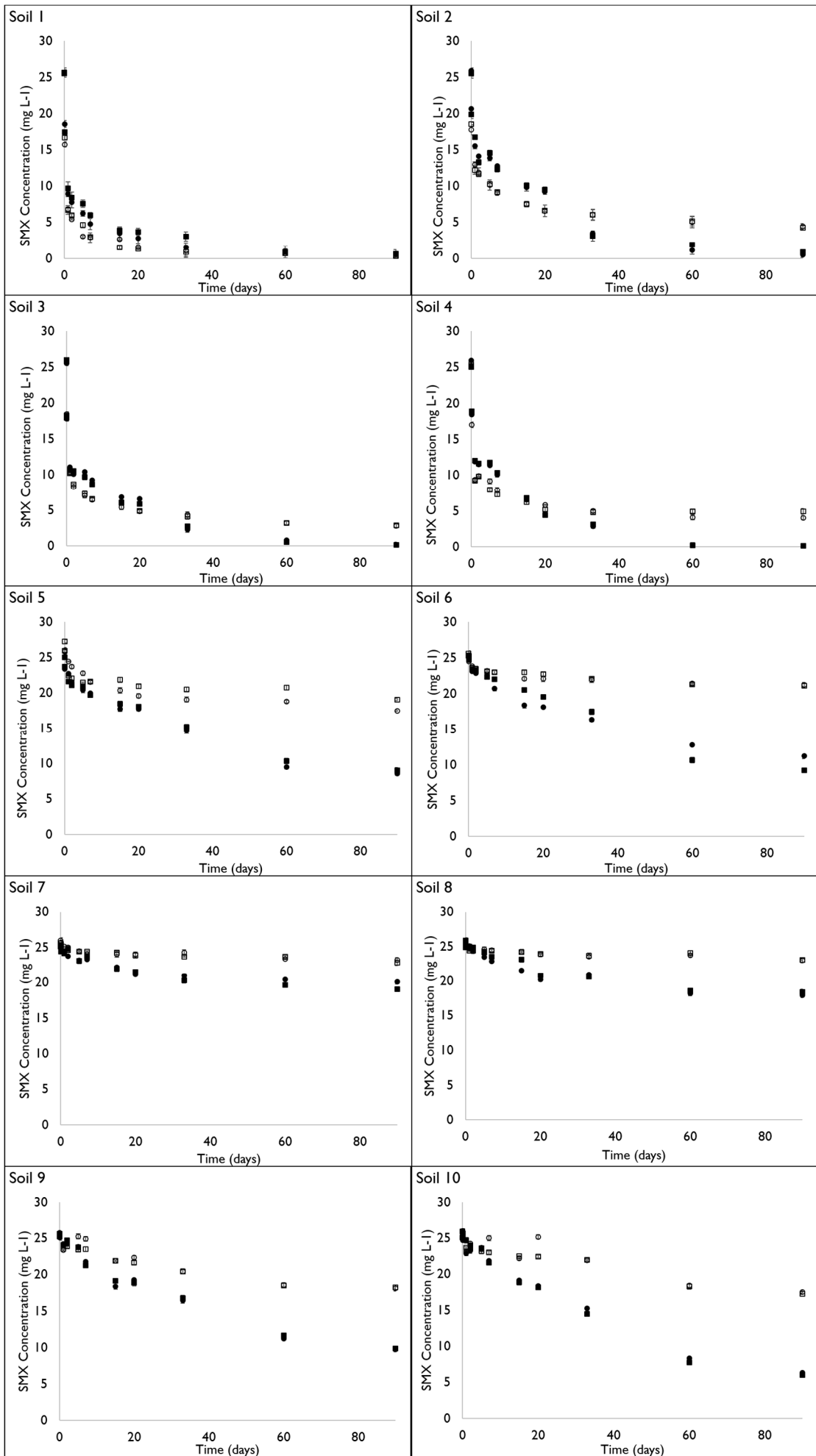
202 **Microbial degradation kinetics and soil's biodegradation capacity of SMX.** Significant and  
203 variable initial decreases in SMX concentration were observed in all soils. This decrease is  
204 generally attributed to the pollutant sorption<sup>20,24</sup> to soil constituents (varying mainly as a function  
205 of the total organic carbon and iron oxides contents of the soils) but not to degradation. Due to their  
206 antibiotic activity, sulfonamides antibiotics (including SMX) were shown to be poorly biodegraded  
207 in the first hours after application to soil, probably due to prior degradation of more readily  
208 biodegradable substances, which could result in competitive inhibition on xenobiotic oxidation<sup>20</sup>.  
209 This is likely to be related to the prior adaptation of the microbial community to the spiked  
210 antibiotic. Consequently, biodegradation kinetics were considered to initiate 24h after the addition  
211 of SMX. Abiotic SMX degradation (hydrolysis and chemical degradation) was similarly assessed in  
212 sterile soils.

213 Figure 2 presents the degradation kinetics of SMX and its linear fit, in sterile and non-sterile  
214 systems. All these kinetics could be correctly described with a first-order kinetic equation, which is  
215 often used to describe the dissipation of organic pollutants in soils<sup>36</sup>. Overall, the coefficients of  
216 determination ( $R^2$ ) were good to very good and ranged between 0.7 and 0.95 and between 0.76 and  
217 0.98 (Table 1) under sterile and non-sterile conditions, respectively. This is in agreement with  
218 several studies conducted in lowland soils showing that SMX biodegradation is generally well  
219 described with a first order decay model, with half-life values ranging between 8 to 40  
220 days<sup>12,18,19,22,24,39,40</sup>. This indicates that SMX is slightly more persistent in the non-sterile upland  
221 soils studied here with SMX half-life ranging from 14 to 346 days, compared to those of lowland  
222 soils. The microbial biodegradation rate constants for SMX were determined from the difference  
223 between the first order rate constant obtained in non-sterile and sterile soil conditions (Table 1).  
224 Biodegradation of SMX was evidenced in soils 2 to 10 as degradation rate constants were higher in  
225 non-sterile systems than in sterile systems. The calculated half-lives of SMX were consistently  
226 always higher in sterile soils (Table 1). In soil 1, the microbial degradation rate constants were  
227 identical in both conditions. This result was reproducible in duplicates indicating that abiotic

228 degradation and sorption are the predominant processes responsible of SMX dissipation. This  
229 shows that in soil 1 SMX degradation is mostly abiotic, suggesting that its microbial community is  
230 poorly adapted to SMX biodegradation due to its distance to human activities and high altitude (see  
231 Fig. 1). This is consistent with the absence of SMX in soil 1 zone (SMX not detectable<sup>8</sup>). In this  
232 soil, SMX dissipated almost completely within 90 days of the experiment under both soil  
233 conditions.

234

235



237 **Figure 2.** Kinetics of SMX degradation ( $\text{mg L}^{-1}$ ) in the ten soils studied (duplicates of sterile and  
 238 non-sterile soils). Empty symbols refer to experimental data in sterile soils (duplicates), filled  
 239 symbols refer to experimental data in non-sterile soils (duplicates).

240

241 **Table 1.** Kinetic parameters of SMX degradation in soil.

Treatment	Soil	First-order rate constant $k$ ( $\text{d}^{-1}$ )	$R^2$	$T_{1/2}$ (d)	Treatment	Soil	First-order rate constant $k$ ( $\text{d}^{-1}$ )	$R^2$	$T_{1/2}$ (d)	Biodegradation rate constant ( $\text{d}^{-1}$ )**	Distance to El Alto city (km)
Sterile condition	1 A	3.00E-02	0.86	23.10	Non-sterile condition	1 A	0.03	0.97	23.10	0	23.20
	1 B	3.00E-02	0.92	23.10		1 B	0.03	0.98	23.10	0	
	2 A	1.10E-02	0.84	63.00		2 A	0.04	0.98	17.33	0.029	7.69
	2 B	1.10E-02	0.8	63.00		2 B	0.034	0.96	20.38	0.023	
	3 A	1.30E-02	0.84	53.31		3 A	0.049	0.98	14.14	0.036	9.53
	3 B	1.20E-02	0.84	57.75		3 B	0.049	0.99	14.14	0.037	
	4 A	1.00E-02	0.81	69.30		4 A	0.054	0.95	12.83	0.044	8.07
	4 B	7.00E-03	0.77	99.00		4 B	0.054	0.94	12.83	0.047	
	5 A	3.00E-03	0.75	231.00		5 A	0.011	0.96	63.00	0.008	10.84
	5 B	1.00E-03	0.84	693.00		5 B	0.01	0.97	69.30	0.009	
	6 A	1.00E-03	0.76	693.00		6 A	0.008	0.96	86.63	0.007	22.99
	6 B	1.00E-03	0.93	693.00		6 B	0.011	0.97	63.00	0.01	
	7 A	7.00E-04	0.7	990.00		7 A	0.002	0.76	346.50	0.0013	29.87
	7 B	7.00E-04	0.92	990.00		7 B	0.002	0.84	346.50	0.0013	
	8 A	8.00E-04	0.8	866.25		8 A	0.003	0.85	231.00	0.0022	32.07
	8 B	5.00E-04	0.75	1386.00		8 B	0.003	0.86	231.00	0.0025	
	9 A	3.00E-03	0.86	231.00		9 A	0.01	0.96	69.30	0.007	51.77
	9 B	3.00E-03	0.92	231.00		9 B	0.01	0.97	69.30	0.007	
	10 A	3.00E-03	0.83	231.00		10 A	0.016	0.98	43.31	0.013	54.99
	10 B	3.00E-03	0.95	231.00		10 B	0.016	0.98	43.31	0.013	

242

243 \* $k$  was determined from equation  $\ln[A] = \ln[A]_0 - kt$ .

244 \*\* Determined from the difference between First-order rate constant  $k$  ( $\text{d}^{-1}$ ) obtained in non-sterile and sterile  
 245 conditions.

246

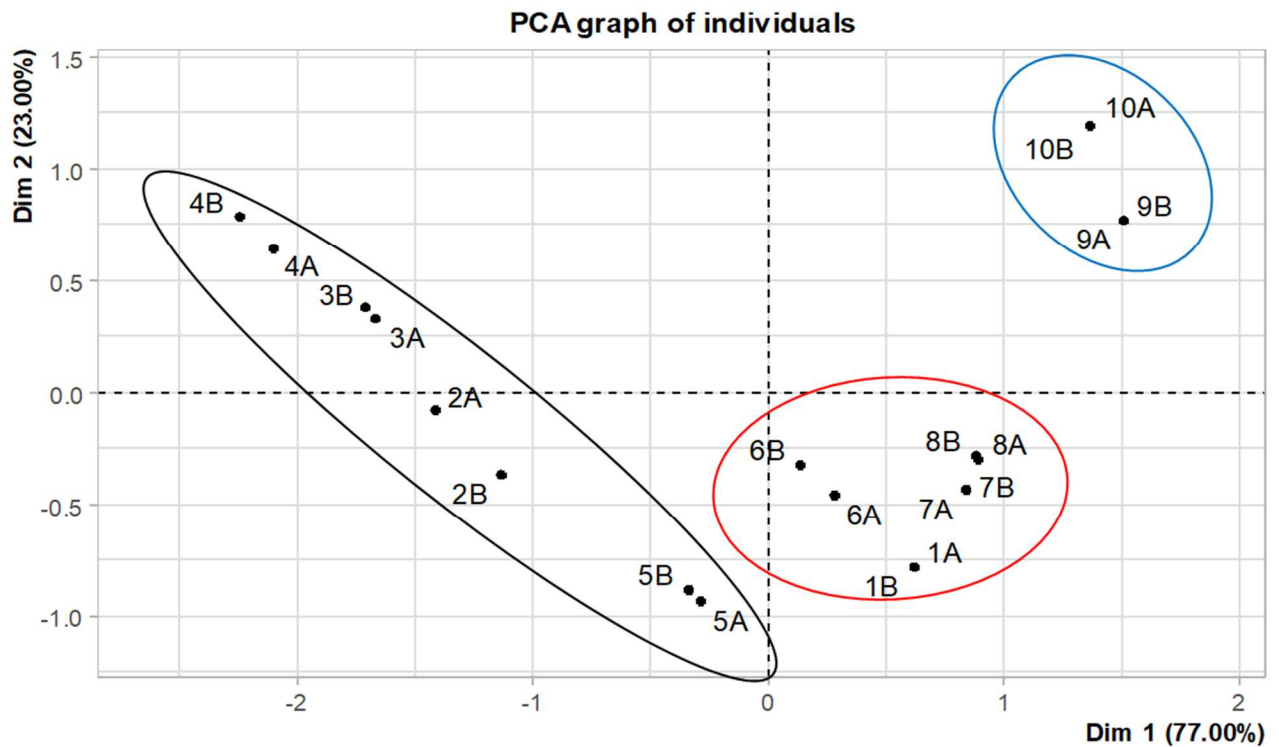
247 Soil 4 showed the highest biodegradation rate as well as the highest OC content (10.3%). Likewise  
 248 soil 4 showed the highest initial SMX concentration ( $18 \mu\text{g kg}^{-1}$ ). The lowest biodegradation rate  
 249 was observed in soil 1, which is OM-rich (8.2 %), but has never been in contact with the antibiotic

250 due to its geographical situation. Soil 2 and 3 also presented high biodegradation rate constant  
251 relative to the other soils (5 to 10), also based on high OC content of 3.6 and 7.29 % (respectively)  
252 than soils 5 to 10 (OC%<2). The relationship between microbial degradation constants and OC  
253 contents by not considering soil 1 show a good relationship ( $R^2= 0.93$ ).

254 These results show that a high level of OC in soil promotes the biodegradation of this antibiotic,  
255 probably related to the higher microbial activity. These results are in agreement with those of Xu et  
256 al. (2011)<sup>18</sup> who showed that SMX biodegradation is significantly increased with increasing humic  
257 acid content in a natural river water-sediment. Our results show that OC-rich soils with pre-adapted  
258 bacterial communities show significant capacities for degradation. However, there is evidence that  
259 SMX is not completely degraded *in situ*, as significant SMX residues have been detected in soils  
260 collected by Archundia et al. (2017)<sup>8</sup>, probably relating to the activity and composition of soil  
261 bacterial community at high-altitude, and also irreversible sorption<sup>35</sup>.

262  
263 The high SMX contamination observed in soil 4<sup>8</sup> probably also favored the pre-adaptation of the  
264 microbial community to SMX and thus its rapid degradation associated with the lower persistence.  
265 The biodegradation constants observed in the soils close to urban areas (soils 2 to 5) were  
266 significantly (*p-value*: 0.0026) higher than those observed in the soils far away from urban areas  
267 (soils 1 and 6 to 10). The PCA results presented in Figure 3 confirm this observation, as all soils are  
268 well distributed along the horizontal axis, which represents the distance from the soil collection site  
269 to the city of El Alto, while the vertical axis represents the biodegradation rate constant. The soils  
270 closest to the city are grouped on the left of Figure 3, while the other soils are grouped on the right.  
271 Soils 9 and 10 show a specific behavior because they are close to Lake Titicaca and far from the  
272 city, but show a high biodegradation rate constant, unlike soils 1 and 6 to 8. This is probably related  
273 to the accumulation of antibiotics in the lake through surface runoff in the basin (Archundia et al.  
274 2017) and on the surrounding soils through frequent grazing of antibiotic-treated cattle. These  
275 results show a clear trend of decreasing SMX biodegradation capacity when moving away from

276 urban areas and human activities (e.g. wastewater discharge areas, domestic and industrial solid  
277 dumping) and not only towards OC contents.



278  
279 Figure 3. Principal component Analysis (PCA) biplot obtained from SMX biodegradation rate  
280 constants (BiodRate) and the distance (Km) of soil sampling points to El Alto city. Statistical  
281 ellipses represent 90% confidence.

282  
283 Overall, our results show that SMX degradation in high-altitude soils is inferior than in low altitude  
284 soils. In studied soils, SMX is degraded by biotic and abiotic processes and remains significantly  
285 persistent, especially in soils that have never been contaminated by this antibiotic. The half-lives  
286 measured in this study in the non-sterile studied soils under suboxic/anoxic conditions were from  
287 12.8 to 346.5 days.

288 In the context of global warming and considering the specific climate characteristics and the  
289 increasing human activities in the catchment, it is likely that both will impact in the future the  
290 persistence of molecules such as antibiotics in soil ecosystems. This is consequently an interesting



291 issue that should receive more attention in the future from both the scientific community and the  
292 public.

293 **Changes in the structure of soil bacterial communities induced by SMX.** Major changes in the  
294 structure of soil bacterial communities of disturbed top layer (0-10 cm) induced by SMX were  
295 studied 30 days after antibiotic contamination in soils 1, 4, 5, 8 and 10 using DGGE fingerprinting  
296 and high-throughput sequencing of PCR-amplified 16s rRNA, *sul1* and *sul2* products. The diversity  
297 and richness indices (from the DGGE) are presented in Table 2.

298 Analysis of the variations in microbial biodiversity revealed by DGGE fingerprints shows that SMX  
299 contamination had a low overall impact on soils microbial structures, as statistical analyses of these  
300 data reveal significant impact only on the soil 1. In soil 1, SMX impacted both the richness and  
301 diversity (Shannon and Simpson indexes) with significant differences in microbial communities  
302 between time 0 (T0) and 30 days post-contamination (30SMX, Table 2) (*p-value*: richness: 6E-03,  
303 Shannon: 2E-03 and Simpson: 1.66E-02). These results are confirmed by the significant difference  
304 in biodiversity indices measured in the control and SMX-contaminated soils at T30 only for soil 1  
305 (*p-value*: richness (5.3E-06), Shannon (4.60E-05), Simpson (1.90E-04)). In all other soils no  
306 significant differences between treatments were observed. Similar results of the impact of SMX  
307 have been observed by Collado et al. (2013)<sup>41</sup> using a DGGE approach in activated sludge.

308 A cluster analysis of all soils results was carried out and presented as a dendrogram (SI. 8).  
309 Although the statistical analysis of the indices calculated with the DGGE data was significant only  
310 in soil 1, the dendrogram revealed impacts for soils 5, 8 and 10. As expected, the dendrogram for soil  
311 1 reveals an immediate impact of SMX because the microbial community structure was changed as  
312 early as one day after SMX spiking. 30 days after SMX contamination, all SMX samples are  
313 grouped separately from the control and T0 samples, indicating a significant impact. The separation  
314 of control samples measured after 1 and 7 days could be attributed to the adaptation of the bacteria  
315 community to the experimental conditions.

316 SMX impact on the microbial communities of soils 5 and 8 remained clearly visible up to 30 days,  
 317 as the corresponding samples (30SMX) appeared separately from the other samples, which were all  
 318 grouped. In soil 10, an impact of SMX was visible only until day 7 (7SMX). No SMX effects are  
 319 detected by clustering in soil 4 (all samples are grouped). Using DGGE fingerprints, Zhang et al.  
 320 (2014)<sup>34</sup> also showed inhibitory effects of SMX (as co-pollutant of estrone and 17 $\beta$ -estradiol) on the  
 321 presence of some bacterial species.

322 The results obtained with high-throughput sequencing are much more sensitive than the DGGE  
 323 results and permitted to show a variable impact of SMX in the different soils. For each soil, 5 000 to  
 324 18 000 filtered and high quality sequences were obtained per sample. Most rarefaction curves  
 325 approached a plateau or were asymptotic; only the T0b rarefaction curve for soil 5 was truncated  
 326 due to the limited number of sequences obtained. Rarefaction curves are presented in SI. 9.  
 327 Bioinformatics analysis of these sequences permitted to calculate several parameters describing the  
 328 microbial diversity of each soil sample (Table 2).

329  
 330 **Table 2.** Diversity parameters of the bacterial communities of five soils of the Katari watershed  
 331 calculated from DGGE fingerprints and DNA sequencing before and after SMX contamination.

332

Soil	Sample	DGGE			High-throughput sequencing (Miseq)			
		Richness	Shannon	Simpson	Equitability (Evenness)	Richness	Shannon	Simpson
1	T0	26	3.13	19.58	0.86	1245.5	6.10	129.7
	30C	34	3.40	26.09	0.80	1005.0	5.54	64.9
	30SMX	21	2.76	12.16	0.63	788.5	4.22	11.8
4	T0	32.5	2.91	11.41	0.86	1322.5	6.16	211.4
	30C	28.5	2.93	14.30	0.80	1126.1	5.62	63.0
	30SMX	30.5	2.83	10.91	0.77	980.3	5.33	67.9
5	T0	37	3.50	28.82	0.88	1114.5	6.16	190.9
	30C	42	3.59	30.59	0.89	1303.0	6.38	219.7
	30SMX	37	3.52	30.57	0.88	1185.0	6.22	198.0
8	T0	27	2.81	9.78	0.88	1795.0	6.62	277.5
	30C	26.5	2.66	9.52	0.89	1726.0	6.64	323.4
	30SMX	23	2.56	8.43	0.80	1306.0	5.74	72.2
10	T0	45	3.39	18.64	0.88	1504.0	6.41	238.1
	30C	34.5	3.15	15.91	0.89	1608.5	6.53	293.3
	30SMX	34.5	2.99	12.06	0.82	1150.5	5.81	97.2

333 Values are averages from three replicates. T0: initial soil sample without any treatment. 30C: 30 days control soils  
334 spiked with water. 30SMX: 30 days after SMX contamination of soils.

335  
336 In Soil 1, a significant decrease in equitability ( $p$ -value:  $5.8E-04$ ), Shannon ( $p$ -value:  $3.5E-03$ ) and  
337 Simpson ( $p$ -value:  $8.4E-03$ ) indices was observed between T0 and T30, confirming the results  
338 obtained with the DGGE fingerprints. Soil 8 showed similar significant changes in biodiversity  
339 indices: equitability ( $p$ -value:  $7.2E-04$ ), Shannon ( $p$ -value:  $8.2E-04$ ) and Simpson ( $p$ -value:  $1.2E-$   
340  $03$ ). Soil 4 also showed significant changes in the indices of richness ( $p$ -value:  $6.7E-03$ ), Shannon  
341 ( $p$ -value:  $2.8E-02$ ) and Simpson ( $p$ -value:  $8.2E-03$ ) at 30 days. Soil 5 did not show statistically  
342 significant variations in bacterial biodiversity. Finally, the calculated indices of SMX-contaminated  
343 soil 10 decreased significantly between 0 and 30 days.

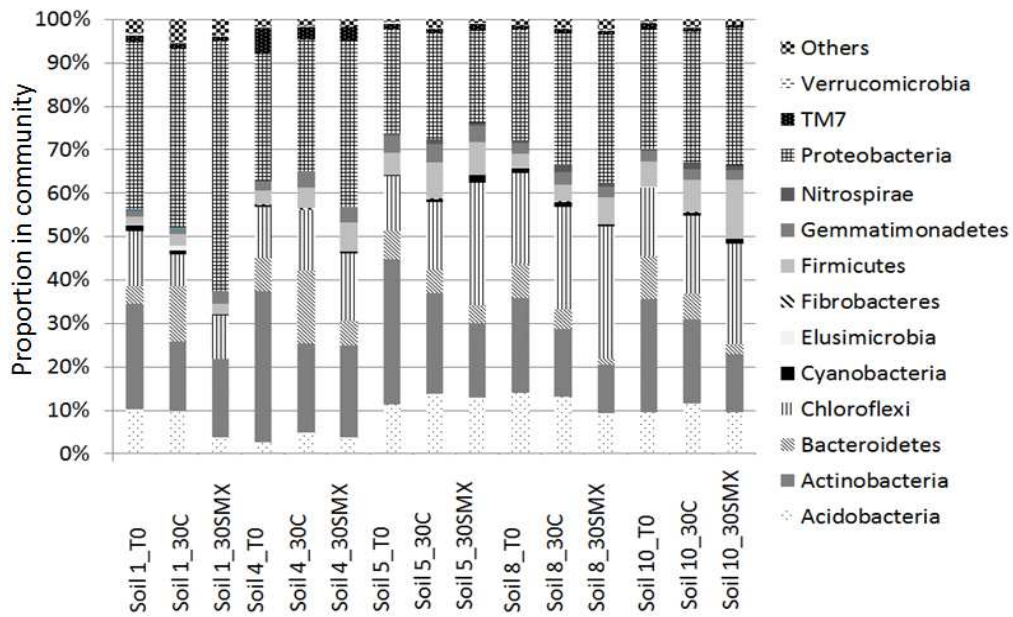
344 These results are in agreement with Demoling et al. (2009)<sup>35</sup> who also observed an impact of SMX  
345 on the structure of the bacterial communities of manure-amended soils, 2 and 5 weeks after  
346 exposure SMX. Cheng (2020)<sup>39</sup> showed that SMX contamination significantly reduced the bacterial  
347 diversity and altered the composition of bacterial and fungal communities in a low altitude soil.  
348 Kang et al. (2018)<sup>44</sup> made similar observations in activated sludge.

349 Differences are related to the difficulty for DGGE to separate all DNA fragments, with low  
350 sequence variation and the limitation of the maximum number of different visible DNA fragments,  
351 which can be separated on the same gel, or to co-migration of DNA fragments. In addition, several  
352 studies have revealed that PCR-DGGE is often limited to the detection of dominant species<sup>45-47</sup>.  
353 Therefore, our results suggest that in soils 4, 5, 8 and 10, the dominant soil bacterial species  
354 successfully tolerated important SMX concentrations.

355 Overall, these results showed that SMX reduced the bacterial diversity of soil bacterial communities  
356 in high-altitude soils as was also observed with low altitude soils<sup>39,43</sup>. Specifically, on the soils of  
357 the Bolivian Altiplano SMX impact is strongly controlled by the historical presence of SMX (pre-  
358 adaptation of microbial populations to SMX) and by the origin of soils with a clear distinction  
359 between agricultural (soils 8 and 10), pristine (soil 1) or urban (soils 4 and 5). Soil 1, which was not

360 initially polluted by SMX and which came from a natural mountain area, is the most sensitive to  
361 SMX contamination, as shown with the DGGE and metabarcoding approaches. Agricultural soils (8  
362 and 10) with intermediate initial concentrations of SMX (0.5 and 1.3 $\mu\text{g kg}^{-1}$ , respectively) were  
363 slightly but significantly impacted by SMX, as evidenced only by the DNA sequencing approach,  
364 which also indicates that SMX preferentially affects non dominant bacterial species undetectable by  
365 the DGGE approach. Finally, soils 4 and 5 with initial SMX concentrations of 0.6 and 18 $\mu\text{g kg}^{-1}$   
366 respectively and originating from urban areas were indifferent to SMX spiking, probably in relation  
367 to a generally well-adapted soil microbial community. These results permitted to decipher the effect  
368 of the land use and of the soil type on SMX degradation. Indeed, our results clearly show that in our  
369 study context, anthropogenic activities are probably the main driver of the antibiotic degradation,  
370 with the development of antibiotic degradation capabilities.

371  
372 **SMX impact on the taxonomic composition of soil microbial communities.** Pyrosequencing of  
373 the 16srDNA genes was performed to explore the bacterial phylogenetic structure of the soils before  
374 antibiotic application (T0), the control soils treated with water after 30 days (30C) and the polluted  
375 soils 30 days after SMX contamination (30SMX). Figure 4 presents the relative abundance of  
376 bacterial phyla in all soils at T0, 30C and 30SMX. At T0 the dominant phyla were *Actinobacter* and  
377 *Proteobacter* in all studied soils. Within *Proteobacter* the most dominant classes were  $\alpha$ ,  $\beta$  and  $\gamma$ -  
378 *Proteobacteria*, while within *Actinobacter*, *Actinobacteria*, *Acidimicrobiia*, and *Thermoleophilia*  
379 were dominant. Similarly, Kumar et al. (2019)<sup>48</sup> showed that Proteobacteria, Acidobacteria and  
380 Actinobacteria were dominant bacteria phyla at a high-altitude soil in India.



381

382 **Figure 4.** Average proportions (n=2) of the dominant bacterial phyla in soils 1, 4, 5, 8 and 10 of the  
 383 Katari catchment before and after SMX contamination (30C: 30 days control soil, 30SMX: 30 days  
 384 SMX soil, T0: initial soil sample without any treatment).

385

386 At T0, the phylum *Proteobacter* was dominant in soils 1 and 8 (relative abundances:38 and 25.4%,  
 387 respectively). Within *Proteobacter*,  $\alpha$ -*Proteobacteria* was the most abundant class in both soils  
 388 (46.7 and 64.2%, respectively). In these two soils the second dominant phylum was *Actinobacter*,  
 389 (24.5 and 22%, respectively), *Actinobacteria* is the most dominant class in this phylum (62.8  
 390 and 64.6%, respectively), while *Thermoleophilia* is the second most dominant class (22 and 16.8%,  
 391 respectively).

392 In soils located near of the WWTP (soils 4 and 5), the initial microbial community (T0) was  
 393 dominated by *Actinobacter* with relative abundances of 35 and 32.5%, respectively, and the second  
 394 more abundant phylum was *Proteobacter* with 29.2 and 26 %, respectively. Within *Actinobacter* the  
 395 most dominant class was *Actinobacteria* with 88 and 63%, respectively, while the most dominant  
 396 class of *Proteobacter* was  $\alpha$ -*proteobacteria* in both soils (56 and 62.5%, respectively). In soil 10,  
 397 which is subjected to flooding due to its proximity to Lake Titicaca, phyla *Actinobacter* and  
 398 *Proteobacter* were present in similar proportions, 27.4 and 26.2, respectively. In this soil, no

399 variation in the relative abundance of the dominant phyla was observed between T0 and 30C  
400 treatments, indicating the absence of incubation effects on microbial community structure, in  
401 contrast to soils 1, 5, 4 and 8.

402 In soil 1, the less influenced by man, *Bacteroidetes* increased and became the third most abundant  
403 phylum. In soil 4 and soil 5 (near of the WWTP) *Proteobacter* increased and became the most  
404 abundant phylum (slightly more abundant than *Actinobacter*). In the agricultural soil studied (soil  
405 8), *Proteobacter* remained dominant and *Chloroflexi* increased and became the second more  
406 abundant phylum after SMX contamination (Fig. 3).

407 Globally, the abundance of *Actinobacter* phylum has decreased in all SMX-contaminated soils (4  
408 and 5), as well as the less dominant *Bacteroidetes* class. *Chloroflexi* increased after 30 days  
409 becoming the most abundant phylum in soil 5 and the second more abundant phylum in soils 8 and  
410 10, probably related to high antibiotic resistance capacity.

411 These results are consistent with observations made by Collado et al. (2013)<sup>41</sup> and Miran et al  
412 (2018)<sup>25</sup> who studied the effects of SMX on the bacterial communities of activated and  
413 acclimatized sludge. These authors observed that *Proteobacter* presented no sensitivity to SMX  
414 while *Actinobacter* is a sensitive phylum. The increase in *Chloroflexi* in soil 5 is probably related to  
415 the high initial concentration of SMX (T0), which may have caused the soil bacterial community to  
416 evolve towards greater tolerance to this antibiotic. In SMX-treated soils, we observed an increase in  
417 *Proteobacter* abundance between T0 and T30 in soils 1 (18%), 4 (7.8%), 8 (9.3%) and 10 (5.9%),  
418 with  $\alpha$ -*proteobacteria* being the most represented class (~68-90%). In all SMX-polluted soils  $\beta$ -  
419 *proteobacteria* decreased significantly after 30 days of exposure to SMX (13.6 to 35.2 % at T0  
420 compared to 3.2 to 13%) in soils, while  $\gamma$ -*proteobacteria* abundance remained almost constant.  
421 Collado et al. (2013)<sup>41</sup> observed an increase in  $\gamma$ -*proteobacteria* after SMX exposure in activated  
422 sludge. The unchanged proportions of  $\gamma$ -*proteobacteria* could relate to the fact that some species  
423 belonging to  $\gamma$ -*proteobacteria* (such as *Thiothrix* sp.) are known to degrade Sulphur-containing  
424 compounds<sup>49,50</sup>.

425 The *Rhodocyclales* order of the  $\beta$ -*proteobacteria* class completely disappeared in SMX-treated  
426 soils, suggesting that these bacteria are sensitive to SMX. This is in disagreement with Collado et  
427 al. (2013) and Kanagawa et al. (2000) observations on activated sludge, reported an increase of  
428 *Rhodocyclales* after SMX contamination<sup>41,51</sup>. Reis et al (2018)<sup>52</sup> observed that *Proteobacter* strains  
429 transformed SMX into equimolar amounts of the lesser toxic derivative N4-acetyl-  
430 sulfamethoxazole.

431 Generally, our results suggest that SMX pollution in high altitudinal soils clearly affect their  
432 microbial community structure. Similar results were observed in lowland soils<sup>39</sup> but with a  
433 difference in affected bacteria as altitude is the major factor affecting the initial biodiversity and  
434 composition of soil microbial communities<sup>48</sup>. A cluster tree representing the microbial communities  
435 of the different soils and treatments is found on SI. 10. It can be seen that soils 1, 4 and 5 are well  
436 separated while soils 8 and 10 are grouped, probably because they exhibit similar microbial  
437 communities with similar response to SMX. This confirms the impact of SMX on soils 1, 5, 8 and  
438 10 (all 30SMX samples are grouped). In soil 4, the effect is less clear because control samples are  
439 mixed with SMX contaminated samples, suggesting a pre-adaptation of these communities to SMX.

440  
441 **Presence and evolution of sulfonamide resistance genes.** The presence of bacterial DNA in all  
442 samples was confirmed by amplification of 16s rRNA gene. qPCR efficiencies for *sul1* and *sul2*  
443 genes in soil 4 were 98.3% and 96.5%, respectively and the R<sup>2</sup> correlation coefficient was 0.99 for  
444 both genes, confirming the quality of the measurements.

445 The sulfonamide resistance genes *sul1* and *sul2* were only detected in soil 4. This could be related  
446 to the limiting high altitudinal conditions (drastic ambient conditions variations and dryness) and  
447 points wastewater as principal source of resistance genes in the watershed<sup>8</sup>.

448 The temporal evolution of the copy number of sulfonamide resistance genes normalized by the  
449 number of 16S rRNA gene in soil 4 could be observed on SI. 11. As expected, this ratio remains  
450 almost constant in soil 4 controls from 0 to 90 days. On the contrary, in the SMX polluted soil, the

451 copy number of both genes increased over time, indicating the induction of SMX resistant bacterial  
452 populations and the lack of resilience as both genes remained at high levels even after complete  
453 dissipation of SMX.

454 Soil 4 is the closest to the WWTP. The presence of both sulfonamide resistance genes can be  
455 explained by the overflowing of waste water and the inlet pipe drilling to irrigate nearby crops with  
456 raw wastewaters<sup>8</sup> and consequently of associated bacteria carrying resistance genes. This happens  
457 during the wet season when the capacity of the treatment plant is exceeded and/or when the local  
458 population illegally diverts wastewater from the inlet of the WWTP to irrigate crops<sup>8</sup>.

459 Such localized SMX pollution has not yet led to the spread of these SMX resistance genes towards  
460 the other soils of the catchment (1, 5, 8 and 10), as these two genes were not detectable by qPCR at  
461 T0 in these soils. This indicates that the presence and level of SMX ARGs are strongly related to  
462 the spread of untreated wastewater to soil surface. These results could also be related to the  
463 extremes conditions encountered in the Altiplano, which probably limit microbial activity and the  
464 development of bacterial resistance to SMX.

465

#### 466 **4 Conclusion**

467 This study aimed at investigating the persistence and impact of the antibiotic Sulfamethoxazole in  
468 10 soils representative of the entire Lake Titicaca basin. The results showed that significant abiotic  
469 and biotic degradation of SMX occurred in all of the high-altitude soils studied, with the exception  
470 of soil 1, which has no history of SMX contamination. However, the observed biodegradation  
471 capacities were generally low, although highly variable, in most of these soils (the significant  
472 presence of SMX in most of the soils in the basin confirms this), compared to the generally quite  
473 rapid degradation of SMX in lowland soils worldwide. The biodegradation rates measured in the 10  
474 soils correlate fairly well with the OC content of the soils and with proximity to urban areas, i.e. to  
475 human activities. High throughput sequencing revealed profound impacts of SMX on soil microbial  
476 communities, except in urban soils, which were not significantly affected by SMX, probably due to



477 their long history antibiotic contamination via uncontrolled wastewater discharges. This continuous  
478 source of antibiotics may have favored soil microbial communities' adaptation to the degradation of  
479 this type of molecule. In addition, the *sul1* and *sul2* sulfonamide resistance genes were detected  
480 only in these highly contaminated soils, suggesting their significant supply by the continuous  
481 wastewater discharge. Surprisingly, SMX spiking in soils did not result in the development of SMX  
482 resistance genes over 90 days in the soils where these genes were initially absent, maybe related to  
483 the low microbial activity of these high altitude soils, unfavorable to horizontal gene transfer. All  
484 these results indicate a quite specific behavior and impact of sulfonamide antibiotics on microbial  
485 communities of highland soils compared to lowland soils.

486

#### 487 **Supporting Information (SI)**

488 **SI.1.** Physico-chemical characteristics of the soils studied. **SI. 2.** SMX chemical analysis. **SI. 3.** 16S  
489 rDNA amplification for DGGE analysis and DGGE analysis procedure. **SI. 4.** Procedure to the  
490 calculation of the diversity index (Shannon, Simpson's) and Richness from the DGGE profiles  
491 measured 0 and 30 days after SMX spiking. **SI. 5.** 16S rRNA amplification by PCR for  
492 Pyrosequencing and bioinformatics analysis. **SI. 6.** Real-time PCR (qPCR) primers and probes  
493 sequences. **SI. 7.** Procedure and the generation of the plasmids carrying the target genes for  
494 calibration curves. **SI. 8.** Dendrograms calculated on the basis of Pearson correlation coefficients.  
495 Sample names are composed as follows: Sampling time in days-type of system (C: control systems  
496 or SMX: SMX systems)-duplicate (a or b). T0: initial soil sample without any treatment. **SI.**  
497 **9.** Rarefaction curves of different soils and treatments after 30 days SMX contact (in duplicated: a  
498 and b). A: Soil 1. B: Soil 4. C: Soil 5, D: Soil 8. E: Soil 10. **SI. 10.** Cluster tree of different soil  
499 microbial communities obtained with the UPGMA algorithm (pondered UNIFRAC). Numbers refer  
500 to different soils. C30 refers to 30 days control soils. SMX30 refers to 30 days SMX soils. a and b  
501 refer to triplicates. **SI. 11.** Temporal evolution of the copy number of *sul1*(left) and *sul2* (right)

502 sulfonamide resistance genes normalized by the number of 16S rRNA gene in Soil 4 of the Titicaca  
503 catchment. Standard deviations were calculated from triplicates.

504

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512

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