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Do antibiotics have environmental side-effects?

Impact of synthetic antibiotics on biogeochemical processes.

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21 **Abstract**

22 Antibiotic use in the early 1900 vastly improved human health but at the same time started an arms race of
23 antibiotic resistance. The wide-spread use of antibiotics has resulted in ubiquitous trace concentrations of many
24 antibiotics in most environments. Little is known about the impact of these antibiotics on microbial processes or
25 “non-target” organisms. This mini-review summarizes our knowledge of the effect of synthetically produced
26 antibiotics on microorganisms involved in biogeochemical cycling. We found only 31 articles that dealt with
27 effects of antibiotics on such processes in soil, sediment, or freshwater. We compare the processes, antibiotics,
28 concentration range, source, environment, and experimental approach of these studies.

29 Examining the effects of antibiotics on biogeochemical processes should involve environmentally relevant
30 concentrations (instead of therapeutic), chronic exposure (*versus* acute) and monitoring of the administered
31 antibiotics. Furthermore, the lack of standardized tests hinders generalizations regarding the effects of antibiotics
32 on biogeochemical processes. We investigated the effects of antibiotics on biogeochemical N cycling,
33 specifically nitrification, denitrification, and anammox. We found that environmentally relevant concentrations
34 of fluoroquinolones and sulfonamides could partially inhibit denitrification. So far, the only documented effects
35 of antibiotic inhibitions were at therapeutic doses on anammox activities. The most studied and inhibited was
36 nitrification (25-100%) mainly at therapeutic doses, and rarely to environmentally relevant.

37 We recommend that firm conclusions regarding inhibition of antibiotics at environmentally relevant
38 concentrations remain difficult due to the lack of studies testing low concentrations at chronic exposure. There is
39 thus a need to test the effects of these environmental concentrations on biogeochemical processes to further
40 establish the possible effects on ecosystem functioning.

41

42

43 **Keywords** Antibiotic, Biogeochemical processes, Environmentally relevant concentration, Microbial
44 ecotoxicology

45 **Background and purposes**

46 Since their discovery in the 1930s, antibiotics have considerably improved human and animal health
47 and agricultural yields. However, their over-use soon caused problems such as ineffectiveness on pathogenic
48 bacteria (Finley et al. 2013) due to the accelerated development of antibiotic resistant bacteria. Moreover, most
49 antibiotics are not metabolized and are released into the environment via urine and feces. Concentrations of
50 antibiotics can reach several mg/kg of sediment, especially in aquatic ecosystems downstream of manufacturing
51 plants (see review in Larsson et al. 2014) or aquaculture farms (*e.g.* Rico et al., 2014). Even in other
52 environments such as river water or sediment, antibiotics often occur at concentrations from ng/L to $\mu\text{g/L}$ or
53 $\mu\text{g/kg}$. These environmentally concentrations are generally too low to inhibit bacterial activity. On the contrary,
54 inhibitory concentrations (see definition in Nordberg et al. 2009) are often referred to as therapeutic
55 concentrations ($\geq 1 \text{ mg/L}$). The effects of environmentally relevant concentrations of antibiotics can
56 fundamentally differ from therapeutic concentrations, enhancing bacterial communication or transcription
57 regulation (Andersson and Hughes 2014, Davies and Ryan 2012). While many unknowns concerning chronic
58 exposure to low doses remain, there is concern that low doses may favor and sustain genes for antibiotic
59 resistance in the environment (Allen et al. 2010, Kümmerer 2009b).

60 Although antibiotics have been detected in many environments, their ecological effects have been
61 poorly investigated, particularly concerning non-target bacteria and their related ecological functions. Antibiotics
62 target bacteria by preventing their growth (bacteriostatic) or killing them (bactericidal). Antibiotics can be
63 natural (*i.e.* produced by micro-organisms in their own habitat) or of synthetic origin. Most antibiotics target one
64 of three bacterial functions: cell-wall biosynthesis, protein synthesis, or DNA replication and repair. Some
65 antibiotics affect a wide range of pathogens (broad spectrum) while others specifically target a group of bacteria
66 (*e.g.* Gram-positive bacteria). At the level of an individual bacterium, antibiotic-resistance can develop by: (1)
67 pumping out the antibiotic, (2) destroying the active compounds of the antibiotic, (3) reprogramming (or
68 camouflaging) the target structure (Schmieder and Edwards 2012). At the ecosystem level (*i.e.* soil, sediment or
69 water), the microbial community and physical habitat can respond to antibiotic exposure or modulate its effects,
70 with large physicochemical differences between ecosystems and consequently strong differences in response to
71 antibiotic exposure. Ecosystems such as soils are extremely physicochemically complex and dynamic,
72 supporting high microbial diversity. Abiotic inactivation of the antibacterial molecules can occur before ever
73 reaching bacteria by interactions with soil organo-mineral compounds (*e.g.* clays), organic matter (Conkle and
74 White, 2012), or reactions such as photodegradation or hydrolysis (Thiele-Bruhn, 2003). A lack of antibiotic
75 effect can be due to antibiotic physicochemical properties such as a strong sequestration and low bioavailability
76 (Rosendahl et al. 2012) and thus depends on the type of environmental matrix (solid or liquid). The antibiotic
77 tolerance of a bacterial community, or more specifically of a bacterial function, may involve a shift in the
78 community structure (composition, richness, density) or depend on the spatial distribution of members of the
79 community. Bacterial biofilms, made up of an aggregation of microbes of different species surrounded by
80 extracellular polymers, are more tolerant than planktonic bacteria because polymers are resistant to penetration
81 of toxic compounds (Campos et al. 2001; Stewart 2002). Furthermore the more tolerant bacteria in the biofilm
82 benefit from the exposure and become the dominant members of the community (Knapp et al. 2008) or tolerant

83 bacteria may develop on the dead remains of the biofilm (Kotzerke et al. 2011, Demoling et al. 2009,
84 Kleineidam et al. 2010, Reichel et al. 2013, Yergeau et al. 2012).

85 Natural microbial communities are characterized by a large functional redundancy, with multiple
86 species able to carry out the same process. Functional redundancy can allow a process to continue during
87 antibiotic exposure despite modifications in community structure. Bacterial communities can also adapt quickly
88 on hourly or monthly time-frames. Only a few studies have considered both the effects on antibiotic exposure on
89 community structure and ecosystem functioning via process rates (Näslund, et al. 2008, Roose-Amsaleg et al.
90 2013, Underwood et al. 2011, Wunder et al. 2013, Yamamura et al. 2014).

91 Biogeochemical functioning of ecosystems relies largely on microbial activity, with ecosystem services
92 such as nutrient cycling, organic matter production and turnover or degradation of pollutants regulated by
93 microbial metabolism (Ducklow 2008). In this mini-review we investigated how environmentally relevant
94 antibiotic concentrations affect biogeochemical functioning and thus disturb ecosystem processes. We were
95 interested to know if antibiotics have environmental side-effects in addition to inducing and sustaining antibiotic
96 resistance. Studies exploring effects of antibiotics on the microbial community structure and biomass will not be
97 discussed here, only those investigating the side-effect on a biogeochemical process. We first briefly described
98 the fate of antibiotics in the environment and the known microbiological effects of antibiotics, and then provide
99 an overview of the impact of antibiotics on biogeochemical processes. In the last section we assessed the effects
100 of antibiotics on the three major processes of the nitrogen cycle: nitrification, denitrification and anammox. We
101 discussed both acute and chronic antibiotic exposures, though we focused on the latter since it is more
102 environmentally representative. We did not include tests directly conducted on purified enzymes, as the effect on
103 enzyme activity is distinct from the effect of antibiotics on bacterial growth or life. We concluded that future
104 studies should include both community analysis and process rates measurements, in order to establish a
105 mechanistic relationship and explain the effects on biochemical processes mediated by antibiotic exposure.

106

107 **Antibiotics in the environment**

108 In the last 20 years, improved analytical capabilities have allowed the detection of antibiotic residues in
109 virtually all natural habitats (Bell et al. 2013, Kümmerer 2009a, Thiele-Bruhn 2003). At least 1,500 publications
110 have addressed antibiotic molecules in the environment. Similarly, the spread of antibiotic resistance in hospitals
111 and in the environment has been extensively discussed and studied with over 850 publications. Many of the
112 studies report an inventory of antibiotic resistance genes (Nesme et al. 2014) and many address bacteria in
113 aquatic environments (for review see Kümmerer 2009b) or soils (Gatica and Cytryn 2013). The resistance of
114 bacterial species used as fecal indicators was also often investigated (Luczkiewicz et al. 2013, Servais and
115 Passerat 2009). However, despite the proliferation of environmental antibiotic studies in general, relatively few
116 have considered the impact of antibiotics on “ecosystem health” (Gu 2014).

117

118

119 **Microbiological effects of antibiotics in the environment**

120 Regarding the effects of antibiotics in the environment, two aspects have to be considered: (1) the type
121 of bacteria exposed to antibiotics in the environment and (2) the range of antibiotic concentrations to which these
122 bacteria are exposed. While antibiotics can be lethal for pathogenic bacteria, little is known regarding their
123 toxicity on microorganisms that are not the intended targets of a particular antibiotic (Nordberg et al. 2009).
124 These non-target organisms comprise the majority of bacteria inhabiting natural environments. The vulnerability
125 of bacteria to antibiotics is evaluated by measuring the minimum inhibitory concentration (MIC) (Andersson and
126 Hughes 2014). A list of antibiotic MIC exists for bacteria and antibiotics of medical interest (European
127 Committee on Antimicrobial Susceptibility Testing, data from the EUCAST MIC distribution website, last
128 accessed 24 March 2015. <http://www.eucast.org>). Because more than 99% of environmental bacteria are not able
129 to be cultured (Amann et al. 1995) their MIC cannot be measured. Furthermore MIC measures acute lethal
130 toxicity (Nordberg et al. 2009), the effect of high levels over short-term periods as revealed by mortality (Crane
131 et al. 2006), of a single strain of a particular species. This strongly contrasts natural environments where chronic
132 exposure occurs over long periods, sometimes representing a substantial portion of a microbial community's
133 lifespan and toxicity is measured by observing mortality, growth or reproduction (Nordberg et al. 2009).

134 Exposure to therapeutic levels of antibiotics can favor resistant phenotypes, representing a serious
135 public health hazard. However, in environmentally relevant concentrations, vulnerable strains can continue to
136 grow, though sometimes at a reduced rate (Andersson and Hughes, 2014). Though some studies have
137 extrapolated the effects of acute, high doses of antibiotics, there are likely non-linear thresholds and emergent
138 behavior of sub-inhibitory doses in the environment. Sub-inhibitory doses of antibiotics can select for bacterial
139 resistance *in vitro* originating from both enrichment of pre-existing antibiotic resistant bacteria and from
140 selection of *de novo* resistant bacteria (Andersson and Hughes, 2014). These doses further cause the acceleration
141 and spread of resistance in bacteria affecting humans and animals. Sub-inhibitory levels of antibiotic on bacterial
142 physiology can cause mutagenesis, virulence, biofilm formation, and horizontal gene transfer recombination, at
143 least *in vitro*. Furthermore, environmentally relevant concentrations of antibiotics ranging from ng- μ g/L or /kg
144 could act as signaling molecules involved in quorum sensing biofilm formation and virulence (Andersson and
145 Hughes 2014).

146

147 **Effects on biogeochemical processes**

148 The consequences of antibiotics on biogeochemical processes are still not well-documented. To the
149 present date (2015, March), we found 31 articles (Table 1) exploring effects of antibiotics on these processes.
150 Identifying general patterns was complicated by the fact that there is no single standardized method to study the
151 effect on biogeochemical processes. Nevertheless, a common way to study antibiotic effects on biogeochemical
152 processes is to reproduce in the laboratory a simple system and then controlling several parameters, notably the
153 antibiotic exposure. Depending on the nature of the environmental sample, different laboratory tests are used,
154 such as batches (homogenization of the sample) or microcosms (intact soil or sediment incubation; Underwood
155 et al. 2011, Yan et al. 2013). Mesocosms and field trials are less often used due to the complexity of their set up
156 (Rosendahl et al. 2012) even though they tend to better reflect chronic exposure conditions in the environment.

157 We found 12 studies from natural aquatic environments, six from waste water treatment plants (WWTP), eight
158 from soils, and five on bacterial enrichments. 77 % of these studies included processes involved in the nitrogen
159 cycle. The non-nitrogen-related studies involved sulfate-reduction (Hansen et al. 1992, Ingvorsen et al. 2003, Liu
160 et al. 2014), pyrene degradation (Näslund et al.2008), iron reduction (Thiele-Bruhn and Beck 2005, Toth et al.
161 2011), arsenic oxidation and reduction (Yamamura et al. 2014), methanogenesis (Conkle and White 2012,
162 Fountoulakis et al. 2004, Liu et al. 2014), and acetate biodegradation kinetics (Wunder et al. 2013). We only
163 found a few studies that examined the effect of antibiotics on several biogeochemical processes (Conkle and
164 White 2012, Kotzerke et al. 2008, Kotzerke et al. 2011, Liu et al. 2014, Rosendahl et al. 2012, Toth et al. 2011).

165 The 12 studies tested a total of 14 antibiotic families with 31 compounds displaying extremely different
166 physicochemical properties that cause differences in target species and antibiotic mechanisms. In the following
167 sections we give a brief overview of the experimental procedure of exposure concentrations and experimental
168 design to compare and review the effects of antibiotics on biogeochemical processes.

169 Across studies, the fate of the antibiotic substance during the experiment was often unconsidered and
170 only 40% of the studies quantified the antibiotic concentration in the experimental medium to determine the
171 effect on a certain process (Ahmad et al. 2014, Campos et al. 2001, Hansen et al.1992, Hou et al. 2015, Kotzerke
172 et al. 2008, Kotzerke et al. 2011, Liu et al. 2014, Rico et al. 2014, Roose-Amsaleg et al. 2013, Rosendahl et al.
173 2012, Thiele-Bruhn and Beck 2005, Yan et al. 2013). Regardless the antibiotic family, these studies revealed
174 considerable loss of antibiotics over the experiment. In the environment, antibiotic degradation includes diverse
175 processes such as biodegradation, evaporation, and sorption to organic matter. For studies where it was reported,
176 antibiotic concentrations decreased by 62 to 100% during the experiments: 66-100% in soil (Kotzerke et al.
177 2008), 62-93% in sediment (Yan et al. 2013), and 64-98% in water (Rico et al. 2014). Although antibiotics have
178 low volatilities and do not tend to bioaccumulate, they show variable adsorption capacities and decay rates (half-
179 lives from hours to months and even to years in a solid matrix). Sorption coefficients of the 14 antibiotics ranged
180 from high (oxolinic acid) to low (sulfamethoxazole) in the 12 studies. Continuous addition of antibiotic could
181 compensate for the antibiotic degradation observed in all studies. Otherwise studies should consider both
182 nominal and measured antibiotic concentrations to determine whether the degradation rates observed in the
183 laboratory and semi-field experiments provide sufficient antibiotic exposure.

184 We assessed whether acute or chronic toxicity was studied though it was rarely explicitly mentioned,
185 (see Table 1). We considered the duration of the experiment, the type of antibiotic supply, and the antibiotic
186 concentration of the exposure.

187 *Duration of the experiments: short-term versus long-term effects*

188 One of the first studies investigating the effect of antibiotics on a functional trait (denitrification) was
189 short-term, on the scale of hours (Costanzo et al. 2005). Short exposure time is problematic for slow-growing
190 bacteria. Generation times of known cultivated ammonia-oxidizing bacteria vary from 8 to 138 hours with a
191 common value of 20 hours (Prosser 1989) and the doubling time of anammox bacteria is 9 days (Strous et
192 al.1999). Under optimal conditions the doubling time for denitrifiers varies between 1.5-1.9 hours (*P.*
193 *denitrificans* and *P. Stutzeri*, Carlson and Ingraham, 1983). However the doubling time of heterotrophic
194 microorganisms in soils is ~ 9 days (Baath et al. 1988). Acute toxicity tests regularly last hours rather than days

195 or weeks. The duration of the exposure of colistin used by Bressan et al. (2013) on ammonia-oxidizing bacteria
196 was 5 h. Schmidt et al. (2012) already stated that “inhibition of cell division or protein biosynthesis may be
197 observed only after test duration of several days”. The importance of incubation time was also shown in the
198 “Substrate Induced Respiration (SIR)” test of Thiele-Bruhn and Beck (2005); no effect was observed after 4 h,
199 whereas respiration was inhibited by both oxytetracycline and sulfapyridine, after 24 h. Short-term experiments
200 that are shorter than the growth rate of the targeted bacteria, therefore, explore the effect of the antibiotic on
201 enzyme activity. Studies regarding short-term effects (Bressan et al. 2013, Costanzo et al. 2005, Hou et al. 2015,
202 Katipoglu-Yazan et al. 2013) can be contrasted to long-term effect studies: from days (Ahmad et al. 2014,
203 Alighardashi et al. 2009, Conkle and White 2012, Fountoulakis et al. 2004, Hou et al. 2015, Ingvorsen et al.
204 2003, Thiele-Bruhn and Beck 2005, Yamamura et al. 2014), weeks (Campos et al. 2001, Cui et al. 2014, Klaver
205 and Matthews 1994, Kotzerke et al. 2008, Kotzerke et al. 2011, Lotti et al. 2012, Rico et al. 2014, Roose-
206 Amsaleg et al. 2013, Toth et al. 2011, Underwood et al. 2011, Wunder et al. 2013, Yan et al. 2013), months
207 (Fernandez et al. 2009, Hansen et al. 1992, Näslund et al. 2008, Rosendahl et al. 2012) to year (442 days by
208 Schmidt et al. 2012). Conducting experiments for extended time periods is difficult because degradable
209 antibiotics, as well as nutrients can be quickly consumed unless continuously added (Alighardashi et al. 2009).

210 *Supply of antibiotic*

211 In most studies (25 out of 31), the antibiotic was added once or several times (Campos et al. 2001;
212 Fernandez et al. 2009; Rico et al. 2014; Schmidt et al. 2012). Only three studies continuously supplied the
213 antibiotic (Wunder et al. 2013, Yan et al. 2013; Roose-Amsaleg et al. 2013). With a single antibiotic addition,
214 the exposure might be insufficient and a lack of effect can be confounded with a lack of exposure of the
215 antibiotic due to degradation. Among the 24 studies using a single addition of the antibiotic, eight quantified the
216 antibiotic. The chronic exposure cannot be ascertained in the other cases. In order to be sure of the exposed and
217 effective antibiotic levels, these should be measured over the course of the experiment.

218 *Level of exposure*

219 Most studies investigating the effect on biogeochemical process used an antibiotic exposure which is
220 therapeutic (mg/L or mg/kg), thus higher than those found in polluted environments. These high concentrations
221 of antibiotic could be used as a positive control in the experiments, but a larger range of antibiotic
222 concentrations, including environmentally relevant ones should be tested. However over half of the studies (17
223 of 31) exclusively tested those therapeutic levels which do not reflect environmental exposures. Inhibitive effects
224 were almost always observed in those studies but were sometimes reversible (Hansen et al. 1992) or temporary
225 (Kotzerke et al. 2011). Among the five antibiotics tested at 1 mg/L by Costanzo et al. (2005), three inhibited
226 (erythromycin, clarithromycin, amoxicillin), whereas two (ciprofloxacin and the mixture amoxicillin and
227 clavulanic acid) did not inhibit denitrification. Similarly, Campos et al. (2001) did not observe any inhibition for
228 one among two antibiotics (chloramphenicol) on nitrification even when applied at 10-250 mg/L. However as
229 explained by Alighardashi et al. (2009) the use of high levels of antibiotic is appropriate when batch experiments
230 are carried out, notably on wastewater.

231 As a conclusion given by Alighardashi et al. (2009), long-term “laboratory-scale” studies using low
232 antibiotic concentrations should be complemented by short-term “batch” experiments that use large doses.

233 Researchers should be aware of what level of antibiotic exposure best suits their research question: acute or
234 chronic in order to give relevant results. Effects of environmentally relevant concentrations on processes need
235 further study, with particular attention to incubation period, the antibiotic of interest, and the environmental
236 matrix.

237

238 Studies on the effects of cocktails or mixtures of antibiotics on biogeochemical processes are even
239 scarcer but of growing concern (Ingvorsen et al. 2003, Schmidt et al. 2012; Wunder et al. 2013). The positive
240 synergistic effect of an antibiotic mixture on the model organism *Vibrio Fisheri* has been demonstrated by
241 Backhaus et al. (2000). Although the low doses of each antibiotic were not toxic individually, the mixture was
242 strongly toxic. A study carried out with a combination of environmentally relevant concentrations ($\mu\text{g/L}$) showed
243 that the simultaneous exposure to three antibiotics (sulfamethoxazole, ciprofloxacin, and erythromycin) had no
244 effect on the degradation of acetate (Wunder et al. 2013). The two other studies tested therapeutic concentrations
245 of antibiotic mixtures and observed expected inhibition. Schmidt et al. (2012) explored the effect of a mixture of
246 ciprofloxacin, gentamycin, sulfamethoxazole, trimethoprim, and vancomycin (0.1-40 mg/L) on chemical oxygen
247 demand and nitrification. The chosen experimental setup for their study simulated conditions of a waste water
248 treatment plant, nitrification was completely inhibited at 40 mg/L. They concluded that typical conditions in a
249 treatment plant would not result in suppression of nitrogen removal. Similarly, Ingvorsen et al. (2003) showed a
250 strong inhibition (90%) of sulfate reduction after a high dose of chloramphenicol and streptomycine, 20 and 100
251 mg/L, respectively.

252 In the natural environment, multiple antibiotics are present as mixtures. Individual antibiotic
253 applications reveal potential mechanisms of inhibition, whereas to determine realistic effects on processes,
254 mixtures should be investigated. In the future, studies regarding this mixture and possible synergistic effect
255 should be considered (notably using existing concepts for the prediction of mixture toxicities, Backhaus et al.
256 2000). Studying multiple antibiotics at environmentally relevant concentrations as well as the possible role of
257 other contaminants (e.g. metals or pesticide) should be tested.

258

259 **Effect of antibiotics on nitrogen cycle**

260 The following sections specifically examine the three most studied biogeochemical processes in the
261 nitrogen cycle: nitrification, anammox, and denitrification. They represent distinct processes in regard to
262 metabolism and microbes. Bacterial denitrification is almost always facultative (Zumft 1997) in contrast to
263 anammox and denitrification. Although denitrifiers form a phylogenetically diverse group (Philippot and Hallin
264 2005) spread among three kingdoms, microorganisms involved in nitrification or anammox have much lower
265 phylogenetic diversity. This difference might affect their vulnerability to antibiotic exposure, with multi-species
266 communities showing higher resilience to antibiotics (Näslund et al. 2008). Furthermore, both nitrifiers and
267 anammox bacteria have single cellular organizations entailing different responses to antibiotic exposure.
268 Nitrification is carried out in two steps by both Gram-negative bacteria (Kowalchuck and Stephen, 2001) and
269 *Archaea* (Treusch et al. 2005) during ammonia-oxidation and only by Gram-negative bacteria during the nitrite

270 oxidation (Spieck and Lipski 2011). *Archaea* are less sensitive to antibiotics than bacteria due to differences in
271 cell envelopes and metabolic processes (Shen et al. 2013). For example, the ammonia oxidizing archaea
272 *Nitrososphaera viennensis* is resistant to streptomycin, kanamycin, ampicillin, and carbenicillin (Tourna et al.
273 2011). Similarly the anammox bacteria, all members of the order Planctomycetales (Strous et al., 1999) are
274 known for their unique membrane lipids. Furthermore, planctomycetes do not have murein in their cell walls, a
275 target of several antibiotics, and are thus resistant towards these compounds (Claus et al. 2000). Furthermore, the
276 anammox process is carried out within an internal organel, the anammoxosome, which may protect the activity
277 from the effects of antibiotics. Among the studies testing antibiotics on N transformations, thirteen publications
278 studied the effect of antibiotics on nitrification, twelve on denitrification, and two on anammox.

279

280

281 (1) *Nitrification (13 studies - 16 antibiotics)*

282 Nitrifiers have been used to determine the effect of antibiotics on nitrification. The ISO 9509 norm tests
283 toxicity by assessing the inhibition of nitrification of activated sludge microorganisms (Juliastuti et al. 2003).
284 Nitrifiers are highly sensitive to inhibitory compounds (including antibiotics) in aerobic environments.
285 Furthermore nitrifiers are used as biosensors to detect the toxicity of molecules present in waste water treatment
286 plants (Alighardashi et al. 2009, Carucci et al. 2006).

287 Out of the 13 studies, nine showed an effect of antibiotics out of which only four (four antibiotics) did
288 not inhibit nitrification, confirming the high sensitivity of nitrifiers at therapeutic antibiotic concentrations. There
289 was no measured inhibition (1) of soil nitrifiers in the studies involving antibiotic-spiked manure added to soil
290 (Toth et al. 2011, Rosendahl et al. 2012) with monensin and difloxacin, respectively; (2) of mixed nitrifying
291 cultures by chloramphenicol (Campos et al. 2001); (3) of ammonia-oxidizers from a mixed culture of nitrifying
292 bacteria by colistin (Bressan et al. 2013). The latter study observed that nitrite-oxidizing bacteria were more
293 susceptible to colistin than ammonia-oxidizers. Except the physicochemical behavior of the antibiotic, Campos
294 et al. (2001) explained the lack of inhibition by a shift in ammonia-oxidizing bacteria (AOB) versus ammonia-
295 oxidizing archaea (AOA). This is in agreement with Schauss et al. (2009) who calculated via modeling an EC50
296 strongly higher for AOA compared to AOB for sulfadiazine.

297 Among the studies showing an effect, only three tested both environmentally relevant and therapeutic
298 concentrations (Rico et al. 2014, Toth et al. 2011, Schmidt et al. 2012). Rico et al. (2014) carried out a chronic
299 exposure of tropical freshwater to a fluoroquinolone (enrofloxacin) in a microcosm. An inhibitive effect of this
300 antibiotic above 100 µg/L was demonstrated whereas they measured NOEC (No-Observed-Effect-Concentration
301 see Nordberg 2009, for definition) of 10 and 1 µg/L from bacterial and archaeal ammonia-oxidizer abundance,
302 respectively. They actually observed a relation between nitrification activity and an antibiotic exposure at 1,000
303 µg/L, which is above environmentally relevant concentration. They explained this by the “high resilience of the
304 whole water–sediment microbial community and a fast recovery from antibiotic exposure”. Toth et al. (2011)
305 measuring only the production of NO₂⁻ on soil chronically exposed to manure spiked with a sulfonamide
306 (sulfadimethoxin) observed inhibition of ammonia-oxidation until 200 µg/kg.

307 To conclude 14 out of the 16 tested antibiotics inhibited nitrification in the environment at therapeutic
308 levels: sulfadiazine, oxytetracycline, ofloxacin, sulfamethoxazole, erythromycin, ciprofloxacin, gentamicin,
309 colistin, trimethoprim, vancomycin, sulfadimethoxine, enrofloxacin, difloxacin, and tetracycline. The inhibition
310 of such therapeutic antibiotic concentrations could entail severe efficiency loss from 25% (sulfadiazine, in soils
311 at 100 mg/kg; Kotzerke et al 2008) to the complete inhibition of nitrification (all in WWTP; see the antibiotic
312 mixture of Schmidt et al. 2012, tetracycline or erythromycin see in Katipoglu-Yazan et al, 2013). Overall
313 environmental relevant concentrations of fluoroquinolones alone or in mixtures were able to inhibit nitrification
314 rates in environmental samples but the majority of the studies tested therapeutic levels.

315

316

317 (2) Denitrification (12 studies - 14 antibiotics)

318 Denitrification comprises four enzymatic steps involving heterotrophic, facultatively anaerobic micro-
319 organisms. However, the effect of antibiotics on the first step (*i. e.* nitrate reduction and nitrite production) was
320 primarily studied while nitrous oxide production was rarely measured (Hou et al. 2015 and Roose-Amsaleg et al.
321 2013). Acute or chronic effects of several antibiotics on denitrification were studied using therapeutic levels
322 (Costanzo et al. 2005; Murray and Knowles, 1999; Kotzerke et al. 2008, 2011). Recently, environmentally
323 relevant antibiotic concentrations have been tested in eight studies. Denitrification rates were inhibited at
324 environmentally relevant doses of sulfamethoxazole (sulfonamide) in soils (Conkle and White 2012; 500 µg/kg)
325 and groundwater (Underwood et al. 2011, µg/L), whereas nitrate reduction rates were not affected in river
326 sediments (Yan et al. 2013; 10 µg/L). An accumulation of nitrite was observed at low concentrations of
327 sulfamethoxazole in the sediment (Yan et al. 2013), suggesting a negative effect of this antibiotic on the
328 microbial community reducing nitrite to nitrous oxide and nitrogen gas. This supports the theory that a less
329 diverse community is more vulnerable than a diverse one. The reduction of oxidized nitrogen species further to
330 gaseous form is carried out by fewer organisms than nitrate reduction (Zumft 1997). Among the other antibiotics
331 tested at environmentally relevant concentrations, only sulfamethazine was able to inhibit denitrification at
332 environmentally relevant concentrations (Ahmad et al. 2014, groundwater, 10 µg/L; Hou et al. 2015, sediment,
333 50 ng/L). All this suggests that sulfonamides (sulfamethoxazole and sulfamethazine) could represent a risk for
334 the ecosystem health at levels detected in natural habitats, as a widespread, broad-spectrum antibiotic with a low
335 sorption potential (Conkle and White, 2012). The denitrification inhibitions measured ranged from 17 to 82% but
336 never completely blocked the denitrification process.

337

338 (3) Anammox (two studies - four antibiotics)

339 Bacteria responsible for the anammox process express several typical cellular traits such as (1) single-
340 or double-membrane-bounded compartments separating their chromosome from the remainder of the cytoplasm,
341 (2) peptidoglycan-free cell walls (Strous et al. 1999), able to adapt to antibiotics. We would expect anammox
342 bacteria to be more resistant to antibiotics due to their different cell structure, though this depends on the mode
343 of action of the antibiotic (*e.g.* they are insensitive to ampicillin; Strous et al. 1999).

344 Two studies (Fernandez et al. 2009; Lotti et al. 2012) investigated the effect of antibiotics on the
345 anammox process exploring both acute and chronic effects of four different antibiotics (oxytetracycline,
346 tetracycline hydrochloride, sulfathiazole and chloramphenicol). Only therapeutic concentrations
347 (100-1,000 mg/L) were tested in WWTP sludges. The anammox process was inhibited in all the assays
348 regardless of the duration of exposure.

349 Anammox plays an important role in WWTPs as well as in natural environments (*e.g.* Dalsgaard et al.
350 2005). Further study should therefore focus on high-risk areas such as recipients of animal farm wastewater or
351 runoff, aquaculture facilities using antibiotics, WWTP receiving hospital wastewater, and antibiotic synthesis
352 facilities.

353 **Conclusions and perspectives**

354

355 Data on the sources, fate, and effects of antibiotics in the environment, indicate that antibiotics may
356 alter several biogeochemical cycles. In addition to being a threat to public health, antibiotics represent a threat to
357 ecosystem functioning and health, even if their toxic effect could often be buffered in complex and diverse
358 ecosystems. Regarding the existing bibliography, antibiotics such as fluoroquinolones or sulfonamides appear as
359 the most noxious compounds among all families of antibiotics. However these molecules were also the most
360 tested, biasing this preliminary conclusion. Furthermore among the different microbial processes in nitrogen
361 cycling, nitrification and anammox appear to be less sensitive to antibiotic exposure (sensitivity at therapeutic
362 concentrations) than denitrification.

363 In addition to ‘ecosystem health’, there is a concern regarding the impact of antibiotics on ecosystem
364 services delivered by environmental bacteria. Though the data gathered in this mini-review do not allow
365 identifying certain molecules to inform usage recommendations, they clearly demonstrate the importance of
366 further investigation of the effects of antibiotics on biogeochemical processes at environmentally relevant
367 concentrations. It should be noted that from a regulatory perspective, environmental effects of pharmaceuticals
368 are not considered in any way in practice. It is crucial to consider the antibiotic impacts on environments such as
369 the accumulation of nitrite in aquatic environments or of nitrous oxide (a strong greenhouse effect gas) due to a
370 possible inhibition of nitrification or denitrification. In watersheds, wetlands, or waste water treatment plants
371 with clear management priorities regarding nitrogen pollution, small effects of antibiotics on nitrogen removal
372 might be very important. So far, establishing the effect of environmentally relevant antibiotic concentrations on
373 processes is not standardized and results are contradictory (see Table 1).

374 A more standardized approach testing the effect of environmentally relevant concentrations of antibiotic
375 at chronic levels (simulating *in situ* conditions) on more or less sensitive biogeochemical functions is needed to
376 allow comparison between studies. We propose that future studies investigating the exposure of antibiotics on
377 biogeochemical processes include chronic exposure, either via continuous supply of antibiotics or repeated
378 additions. Furthermore experimental design in aquatic systems can involve a mixed batch set-up mimicking
379 environmental conditions. The effect of antibiotics in soil or sediments should preferably be carried out in
380 microcosm, mesocosm, or field studies. In the latter, a survey of the administered compounds is recommended to
381 rank antibiotics regarding their increasing ecological impact to allow the prioritization of regulation depending
382 on different usages. Besides the type of antibiotic, other biogeochemical processes should be considered, in
383 addition to N cycling processes, such as those involved in metal transformation or degradation of pollutants.
384 Furthermore, case studies investigating the effect of antibiotics on biogeochemical processes should determine
385 the effect of these pharmaceuticals at a larger scale, involving a comprehensive interdisciplinary approach,
386 including hydrology (transport), chemistry (nutrients, pollutants, and pharmaceuticals), and biology (process
387 rates and involved organisms).

388

389

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573

574 **Table 1.** List of observed effects of antibiotic on biogeochemical processes in environmental samples; A and C,
575 mean acute and chronic effect, respectively; An exposure was designed as chronic if the duration of the exposure
576 reached a value higher than the life-span of the targeted organisms and eventually if the level of exposure was
577 low (ng-µg/L or kg). WWTP designs waste water treatment plant and S and C design single and continuous
578 addition, respectively. Processes from the nitrogen cycle are in bold. ¹Calculated by the authors. ²Depending on
579 soil type tested.

Microbial Process	Chronic /Acute	Antibiotic					Effects (approximate % change)	Effective concentration	Environment	Reference
		Molecule	Nominal concentration	Addition	Duration	Quantification (loss)				
Acetate biodegradation	C	sulfamethoxazole, erythromycin, and ciprofloxacin (mixture)	0.33-3.33 µg/L	C	weeks	no	no effect	-	water/biofilm	Wunder et al. 2013
Anammox	A	tetracycline hydrochloride	100-1,000 mg/L	S	hours	no	inhibition (30%)	100 mg/L	WWTP	Fernandez et al. 2009
Anammox	C	tetracycline hydrochloride	50 mg/L	C	months	no	reversible inhibition (40%)	50 mg/L	WWTP	Fernandez et al. 2009
Anammox	A	sulfathiazole	100-1,000 mg/L	S	hours	no	inhibition (up to 50%) decrease with the duration of exposure	450 mg/L	WWTP	Lotti et al. 2012
Anammox	C	sulfathiazole	100 and 500 mg/L	S	weeks	no	inhibition (final decrease of 50%)	100 mg/L	WWTP	Lotti et al. 2012
Anammox	A	oxytetracycline	100-1,000 mg/L	S	hours	no	inhibition (up to 20%) decrease with the duration of exposure	200 mg/L	WWTP	Lotti et al. 2012
Anammox	C	oxytetracycline	100 and 500 mg/L	S	weeks	no	inhibition (final decrease of 25%)	100 mg/L	WWTP	Lotti et al. 2012
Anammox	A	chloramphenicol	250-1,000 mg/L	S	hours	no	inhibition (40%)	250 mg/L	WWTP	Fernandez et al. 2009
Anammox	C	chloramphenicol	20 mg/L	C	months	no	irreversible inhibition (up to 80%)	20 mg/L	WWTP	Fernandez et al. 2009
Arsenate reduction	C	tetracycline	50 mg/L	S	days	no	accelerated	50 mg/L	sediment	Yamamura et al. 2014
Arsenate reduction	C	lincomycin	50 mg/L	S	days	no	minor effect	50 mg/L	sediment	Yamamura et al. 2014
Arsenate reduction	C	erythromycin	50 mg/L	S	days	no	minor effect	50 mg/L	sediment	Yamamura et al. 2014

Arsenate reduction	C	chloramphenicol	50 mg/L	S	days	no	no effect (aerobic), inhibition (anaerobic)	50 mg/L	sediment	Yamamura et al. 2014
Arsenate reduction	C	ampicilline	50 mg/L	S	days	no	partial inhibition	50 mg/L	sediment	Yamamura et al. 2014
Arsenite oxidation	C	tetracycline	50 mg/L	S	days	no	delayed	50 mg/L	sediment	Yamamura et al. 2014
Arsenite oxidation	C	lincomycin	50 mg/L	S	days	no	minor effect	50 mg/L	sediment	Yamamura et al. 2014
Arsenite oxidation	C	erythromycin	50 mg/L	S	days	no	minor effect	50 mg/L	sediment	Yamamura et al. 2014
Arsenite oxidation	C	chloramphenicol	50 mg/L	S	days	no	inhibition	50 mg/L	sediment	Yamamura et al. 2014
Arsenite oxidation	C	ampicilline	50 mg/L	S	days	no	indeterminable	50 mg/L	sediment	Yamamura et al. 2014
Chemical oxygen demand removal	C	ciprofloxacin, gentamicin, sulfamethoxazole, trimetoprim, vancomycin (mixture)	0.1-40 mg/L	C	months	extrapolation	reversible inhibition (25%)	30 mg/L	WWTP	Schmidt et al. 2012
Denitrification	C	ciprofloxacin	10-50 mg/L	S	weeks	yes, biotransformation	inhibition (25%)	50 mg/L	enrichment cultures	Liu et al. 2014
Denitrification	C	sulfamethoxazole	1.2 µg/L-0.5 g/L	S	weeks	no	inhibition (47%)	1.2 µg/L	groundwater	Underwood et al. 2011
Denitrification	C	sulfamethazine	0.01-1 mg/L	S	days	yes	inhibition of nitrate removal (17%)_and nitrite production (82%) ¹	0.01 mg/L_1 mg/L	groundwater	Ahmad et al. 2014
Denitrification	C	chlortetracycline	0.01-1 mg/L	S	days	yes, no degradation	inhibition of nitrate removal (15.4%)_and nitrite production (31%) ¹	1 mg/L_1 mg/L	groundwater	Ahmad et al. 2014
Denitrification	C	flumequine	0.1 µg/L-50 mg/L	C	weeks	yes (62%)	inhibition (41%)	50 mg/L	sediment	Yan et al. 2013

Denitrification	C	sulfamethoxazole	0.2 µg/L-50 mg/L	C	weeks	yes (93%)	inhibition (39%)	50 mg/L	sediment	Yan et al. 2013
Denitrification	C	vancomycine	0.3 µg/L-0.2 mg/L	C	weeks	no	no effect	-	sediment	Yan et al. 2013
Denitrification	C	tetracycline	0.9 µg/L-7 mg/L	C	weeks	yes (92%)	no effect	-	sediment	Roose-Amsaleg et al. 2013
Denitrification	A	sulfamethazine	0.05-100 µg/L	S	hours	yes	inhibition (20-30%)	50 ng/L	sediment	Hou et al. 2015
Denitrification	A	erythromycin	1 mg/L	S	hours	no	inhibition (around 37%)	1 mg/L	sediment	Costanzo et al. 2005
Denitrification	A	clarithromycin	1 mg/L	S	hours	no	inhibition (around 37%)	1 mg/L	sediment	Costanzo et al. 2005
Denitrification	A	ciprofloxacin	0.1-1,000 µg/L	S	hours	no	no effect	-	sediment	Costanzo et al. 2005
Denitrification	A	amoxicillin, clavulanic acid	1 mg/L	S	hours	no	no effect	-	sediment	Costanzo et al. 2005
Denitrification	A	amoxicillin	1 mg/L	S	hours	no	inhibition (around 37%)	1 mg/L	sediment	Costanzo et al. 2005
Denitrification	C	tetracycline	1-1,000 µg/kg	S	days	no	no effect	-	soil	Conkle and White, 2012
Denitrification	C	sulfamethoxazole	1-1,000 µg/kg	S	days	no	inhibition	500 µg/kg	soil	Conkle and White, 2012
Denitrification	C	ciprofloxacin	1-1,000 µg/kg	S	days	no	no effect	-	soil	Conkle and White, 2012
Denitrification	C	sulfadiazine	10 and 100 mg/kg	S	weeks	yes (66 -100%)	inhibition (around 70%)	10 mg/kg	soil	Kotzerke et al. 2008
Denitrification	C	difloxacin, sarafloxacin	7-12 µg/kg	S	month	yes	no effect	-	soil	Rosendahl et al. 2012
Denitrification	C	difloxacin	1-100 mg/kg	S	weeks	yes	inhibition (50%)	10 mg/kg	soil	Kotzerke et al. 2011
Denitrification	A	chloramphenicol	100-2,000 mg/L	S	hours	no	inhibition (19-41%) ²	100 mg/L	soil	Murray and Knowles, 1999
Iron reduction	C	sulfapyridine or oxytetracycline	0.02-500 mg/kg	S	days	yes	inhibition (10%) ¹	ED 10 = 0.003_7.35 µg/g	soil	Thiele-Bruhn and Beck, 2005
Iron reduction	C	sulfadimethoxin	25-200 µg/kg	S	weeks	no	inhibition (>95%)	25 µg/kg	soil	Toth et al. 2011
Iron reduction	C	monensin	10-100 µg/kg	S	weeks	no	reversible	10-100 µg/kg	soil	Toth et al.

Methanogenesis	C	ciprofloxacin	10-100 mg/L	S	weeks	yes, no biotransformation	inhibition (40%)	80 mg/L	enrichment cultures	2011 Liu et al. 2014
Methanogenesis	A	sulfamethoxazole	10-400 mg/L	S	days	no	inhibition	IC50 > 400 mg/L	enrichment cultures	Fountoulakis et al. 2004
Methanogenesis	A	ofloxacin	10-400 mg/L	S	days	no	inhibition	IC50 = 334 mg/L	enrichment cultures	Fountoulakis et al. 2004
Methanogenesis	C	sulfamethoxazole	1-1,000 µg/kg	S	days	no	stimulation (30%)	500 µg/kg	soil	Conkle and White, 2012
Methanogenesis	C	tetracycline	1-1,000 µg/kg	S	days	no	no effect	-	soil	Conkle and White, 2012
Methanogenesis	C	ciprofloxacin	1-1,000 µg/kg	S	days	no	inhibition (25%)	1 µg/kg	soil	Conkle and White, 2012
Nitrification	A	colistin	0.3-300 mg/L	S	hours	no	no effect on nitrite oxidation	-	mixed culture	Bressan et al. 2013
Nitrification	A	colistin	0.3-300 mg/L	S	hours	no	inhibition of ammonia-oxidation (correlation)	0.3-300 mg/L	mixed culture	Bressan et al. 2013
Nitrification	C	chloramphenicol	10-250 mg/L	C	weeks	yes	no effect	-	mixed culture	Campos et al. 2001
Nitrification	C	oxytetracycline hydrochloride	10-250 mg/L	C	weeks	yes	inhibition (50%)	250 mg/L	mixed culture	Campos et al. 2001
Nitrification	A	ofloxacin or sulfamethoxazole	2-10 mg/L	S	hours	no	inhibition (75 and 60%)	6 mg/L for each	pure culture	Dokianakis et al. 2004
Nitrification	C	sulfadiazine	10 and 100 mg/kg	S	weeks	yes (66-100%)	inhibition (around 25%)	100 mg/kg	soil	Kotzerke et al. 2008
Nitrification	C	oxytetracycline	12.5-75 mg/L	S	week	no	inhibition (50%) ¹	EC 50 = 8.60-26.96 mg/L	water	Klaver and Matthews, 1994
Nitrification	A	erythromycin	1-267 mg/L	S	days	no	inhibition (72%)	>20 mg/L	WWTP	Alighardashi et al. 2009
Nitrification	C	ciprofloxacin, gentamicin, sulfamethoxazole, trimetoprim, vancomycin (mixture)	0.1-40 mg/L	C	months	extrapolation	complete inhibition	40 mg/L	WWTP	Schmidt et al. 2012
Nitrification	A	tetracycline	50 or 200	S	hours	no	complete	200 mg/L	WWTP	Katipoglu-

Nitrification	A	erythromycin	50 or 200 mg/L	S	hours	no	complete inhibition	50 mg/L	WWTP	Yazan et al. 2013 Katipoglu-Yazan et al. 2013
Nitrification	C	enrofloxacin	1-1,000 µg/L	C	weeks	yes (70-98%)	inhibition	NOEC calculated by authors: bacterial and archaeal ammonia oxidizers to enrofloxacin 10 and 1 µg/L, respectively)	freshwater	Rico et al. 2014
Nitrification	C	sulfadimethoxin	25-200 µg/kg	S	weeks	no	inhibition (60%)	200 µg/kg	soil	Toth et al. 2011
Nitrification	C	monensin	10-100 µg/kg	S	weeks	no	no effect	-	soil	Toth et al. 2011
Nitrification	C	difloxacin, sarafloxacin	7-12 µg/kg	S	month	yes	no effect	-	soil	Rosendahl et al. 2012
Nitrification	C	difloxacin	1-100 mg/kg	S	weeks	yes	inhibition (around 30%)	10 mg/kg	soil	Kotzerke et al. 2011
Nitrification	C	ciprofloxacin	1-50 mg/kg	S	weeks	no	stimulation (20%)	1 mg/kg	soil	Cui et al. 2014
Organic carbon removal	A	tetracycline	50 or 200 mg/L	S	hours	no	inhibition (25 and 71%)	50 and 200 mg/L	WWTP	Katipoglu-Yazan et al. 2013
Organic carbon removal	A	erythromycin	50 or 200 mg/L	S	hours	no	inhibition (22 and 26%)	50 and 200 mg/L	WWTP	Katipoglu-Yazan et al. 2013
PAH (Pyrene) degradation	C	ciprofloxacin	20-2,000 µg/L	S	month	no	inhibition (66%)	1,000 µg/L	sediment	Näslund et al. 2008
Sulfate reduction	C	oxytetracycline, oxolinic acid, flumequine	400, 100, 100 mg/kg	S	months	yes dissipation	reversible inhibition (90%)	400, 100, 100 mg/kg	sediment	Hansen et al. 1992
Sulfate reduction	A	chloramphenicol + streptomycine	20 and 100 mg/L	S	days	no	inhibition (90%)	20 mg/L chloramphenic	WWTP	Ingvorsen et al. 2003

Sulfate reduction	C	ciprofloxacin	10-80 mg/L	S	weeks	yes, no biotransformation	reversible inhibition	ol + 100 mg/L streptomycine 10-80 mg/L	enrichment cultures	Liu et al. 2014
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