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Antibiotic Pollution in Surface Fresh Waters: Occurrence and Effects

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1 **Abstract**

2 Worldwide, antibiotic usage exceeds 100,000 tons per year and there is increasing concern
3 over the fate of these substances. Antibiotics are ubiquitous in the environment and
4 significant concentrations have been detected in fresh waters. In this review, we highlight
5 important aspects of antibiotic pollution in fresh waters: that concentrations of antibiotics in
6 the environment are substantial, that micro-organisms are susceptible to this, that bacteria can
7 evolve resistance in the environment, and that antibiotic pollution affects natural food webs
8 while interacting with other stressors; which taken together poses a number of challenges for
9 environmental scientists.

10 In the literature, we found examples of considerable antibiotic pollution in fresh waters. In
11 the Americas, antibiotic concentrations of up to 15 µg/L have been measured; with higher
12 concentrations reported from European and African studies (over 10 µg/L and 50 µg/L
13 respectively), and in Asian-pacific countries concentrations over 450 µg/L have been
14 detected.

15 While these concentrations might not be deemed harmful to humans, non-target freshwater
16 organisms could be affected by them. Bioassays show that some of the antibiotics found in
17 surface waters affect microbes at concentrations below 10 µg/L. Among the most potent
18 antibiotics are those that prevail in streams and rivers in these concentrations, such as
19 ciprofloxacin. Sub-lethal concentrations might not kill prokaryotes but contribute to increased
20 bacterial resistance and change the composition of single-celled communities, as
21 demonstrated in laboratory experiments. This has implications for the microbial food web
22 (e.g. interactions among and between bacteria and their protozoan consumers) and by
23 extension, larger organisms and ecosystem health.

24 The fact that the effects of antibiotics are extremely context-dependent represents a
25 challenge, particularly for in vitro research. We suggest future research avenues, taking into

- 1 account food web experiments, antibiotics interacting with one another (and other stressors)
- 2 and discuss how these can help to answer multi-layered research questions.

1 **1. Introduction**

2 Antibiotics are antimicrobial drugs that kill or inhibit the growth of bacteria. There are
3 several kinds of antibiotics and they can be classified based on their action mechanisms or
4 chemical structure. They have been used in large quantities for some decades and resistance
5 of pathogens to antibiotics has long been a focal point of research in clinical settings and, in
6 more recent years, in environmental research. The parent compounds of antibiotics, or their
7 metabolites, can be stable enough to bypass water treatment processes and leak into the
8 environment (Kümmerer, 2009), and although diluted by a factor of over one million
9 compared to concentrations in the human body (Jjemba, 2006), the antibiotics dispersed in
10 the environment could have important consequences for both human health and ecosystems.

11 While antibiotics are specifically administered therapeutically to treat pathogen
12 infection and/or prophylactically to boost yields (in livestock farming), non-target organisms
13 (that are part of ecological processes, such as nutrient cycling or degradation of pollutants)
14 are inevitably exposed once antibiotics reach the environment (Flaherty and Dodson, 2005).
15 The resulting concentration of antibiotics entering aquatic systems can interact with the
16 native organisms and can, for example, begin to change the microbial community structure
17 and genetics (e.g., prevalence of resistance genes) (Singer et al., 2016). Prokaryote
18 microalgae (cyanobacteria) can be particularly vulnerable to antibiotics (Cabello, 2006) and
19 continuous exposure of the environment to antibiotics can enhance the selection of resistant
20 bacterial strains, which include potential pathogens (Kümmerer, 2004). Another, much less
21 studied hypothesis is that antibiotics could also have indirect cascading effects on species that
22 are not directly affected by antibiotics but feed on prokaryotes.

23 This review firstly provides a synopsis of antibiotic concentrations in fresh waters
24 (especially running water – i.e. streams and rivers), highlighting the dominant antibiotics in
25 the open water (as opposed to the sediment). Secondly, we cross-referenced these antibiotic

1 concentrations with available literature on aquatic organisms' susceptibility in bioassays to
2 estimate whether concentrations found in the field affect freshwater organisms. Thirdly, we
3 look at available data on antibiotic resistance in natural microbial communities in fresh water
4 settings. Finally, we aim to place this evidence into the context of more complex scenarios,
5 such as potential effects of antibiotic pollution for real food webs (involving a whole suite of
6 organisms from prokaryotes to single celled eukaryotes to metazoans) or interactions of
7 antibiotics with each other and temperature. We suggest future research avenues including
8 food web experiments that provide a realistic and rigorous way to demonstrate how micro-
9 pollutants affect microbial assemblages and by extension entire communities.

10 In the following, we build our arguments on existing reviews on this topic such as
11 Carvalho and Santos (2016) and Segura et al. (2015) (both on antibiotic concentrations in
12 fresh waters), Grenni et al., (2018) (antibiotic effects on bacteria in the environment), and on
13 reviews on micro-pollutants in running waters (Hughes et al., 2012; Monteiro and Boxall,
14 2015). We want to highlight that there is a substantial body of literature on antibiotics in the
15 environment and that we chose examples from that literature that we believe to be relevant
16 and representative.

17

18 **2. The sources and fate of antibiotic pollution in fresh water**

19 There are three major pathways for antibiotics into fresh waters: (1) effluents from
20 Wastewater Treatment Plants (WWTPs), (2) chemical manufacturing plants and (3) animal
21 husbandry and aquaculture (Kümmerer, 2009; Singer et al., 2016). Wise (2002) estimated
22 antibiotic consumption worldwide to lie between 100,000 and 200,000 ton per annum, with
23 approximately 50% used for veterinary medicine and as growth promoters. Global
24 antibiotic consumption by humans alone increased by 36% between 2000 and 2010 which
25 illustrates that antibiotic pollution is an ever-growing problem (Van Boeckel et al., 2014).

1 In general, 50-80% of total parent compounds is excreted through urine and faeces:
2 higher excretion rates are observed for ciprofloxacin (50 to 80%) and tetracycline (80 to
3 90%), while lower excretion rates are observed for erythromycin (5 to 10%),
4 sulfamethoxazole (15 to 30%) or clarithromycin (25%) (Mompelat et al., 2009). In
5 wastewater disposal systems, antibiotics can be eliminated through retention in sludge and/or
6 biotic and abiotic degradation. However, breakdown is often incomplete, and antibiotics and
7 their metabolites end up entering the environment (mainly fresh waters such as streams and
8 rivers). The proportion of the parent compound excreted via WWTP effluents can differ
9 greatly among antibiotics and the process used (Monteiro and Boxall, 2015) but Kümmerer
10 and Henninger (2003) found that approximately 70% of the consumed amount of antibiotics
11 was excreted unchanged from hospitals and households into effluents in Germany. The
12 sorption behaviour of antibiotics (i.e. what they preferentially absorb onto the solid phase)
13 can also be very complex. For example, fluoroquinolones become highly enriched in sewage
14 sludge (Giger et al., 2003) while in the case of sulfonamides, it has been found that
15 elimination by sorption to soil particles is a significant process, meaning that they are
16 preferentially found in wastewater (Heise et al., 2006).

17 Remarkable concentrations of micropollutants are detected in effluent wastewaters.
18 For example, in hospital effluents near the Ter River in Spain, ofloxacin and ciprofloxacin
19 have been found at concentrations of over 13 µg/L (Rodriguez-Mozaz et al., 2015). Östman
20 et al. (2017) in Sweden found ciprofloxacin and erythromycin with concentrations of 1.41
21 µg/L and 0.47 µg/L in incoming sewage water and 0.06 µg/L and 0.35 µg/L in treated
22 effluent respectively. From WWTP effluents to The Cache la Poudre River in northern
23 Colorado ampicillin and oxacillin have been reported at concentrations of 86 µg/L and 95
24 µg/L respectively (Cha et al., 2006) and from WWTP effluents to the Msunduzi River in
25 South Africa ampicillin and ciprofloxacin were found at concentrations of 9 µg/L and 27

1 $\mu\text{g/L}$ respectively (Agunbiade and Moodley, 2016). Much higher concentrations are possible:
2 in the effluent from a wastewater treatment plant serving about 90 bulk drug manufacturers in
3 Patancheru, near Hyderabad in India, ofloxacin and ciprofloxacin have been found at
4 concentrations up to $160 \mu\text{g/L}$ and $31,000 \mu\text{g/L}$ respectively (Larsson et al., 2007). This area
5 can be considered as an exception as there is a very large number of industries congregated in
6 a limited area (Larsson, 2014) but nonetheless, the concentrations of antibiotics are
7 alarmingly high.

8 WWTP effluents and run-off from the terrestrial landscape are the main sources for
9 antibiotic pollution (and other micropollutants) in the open water of streams and rivers. We
10 mainly focus on antibiotic concentrations that can be found in the open water of streams and
11 rivers because data for the open water are more readily available. However, it is worth
12 mentioning that micropollutants such as antibiotics attenuate in the sediment (Peralta-
13 Maraver et al., 2018b; Robertson and Wood, 2010), and that sediment acts as a mechanical
14 and biogeochemical filter ('bioreactor') (Lewandowski et al., 2011). Clearly, the sediment
15 has a critical role in pollution attenuation and removal rates of emerging micropollutants
16 (Peralta-Maraver et al., 2018b, 2018a).

17

18 **3. Antibiotic concentrations in open water of streams and rivers**

19 Previous reviews on concentrations of antibiotics in freshwaters have been published
20 (Carvalho and Santos, 2016; Segura et al., 2015). Our review focusses specifically on studies
21 that reported antibiotic concentrations in surface waters of streams and rivers worldwide, in
22 areas away from WWTP outfalls. In these studies, most of the antibiotic concentrations were
23 measured by targeted liquid chromatography–mass spectrometry (LC–MS), an analytical
24 technique that combines the physical separation capabilities of liquid chromatography with a
25 high sensitivity mass spectrometer (Dinh et al., 2011; García-Galán et al., 2010) (Table 1).

1 We selected studies where concentration values were higher than the limits of quantification
2 and the sample was clearly identified as surface water.

3 We found 54 studies in total, from 28 different countries (Table 1). The countries with
4 the highest number of reports about the occurrence of antibiotics were Spain and China
5 (Table 1). In Europe, some antibiotics were measured at concentrations above 10 µg/L such
6 as sulfapyridine (Díaz-Cruz et al., 2008) and sulfamethoxazole (Ginebreda et al., 2010), both
7 in Spain (Table 1). Based on the data available to us, Spain is the European country with the
8 highest number of studies reported (15) and with the highest concentration of antibiotics (1.3
9 µg/L on average found in surface waters - for European full data see the electronic
10 supplementary material, table S1).

11 In the Americas, antibiotic concentrations of around 2 µg/L or less were found in
12 fresh waters, except for sulfadimethoxine, found at 15 µg/L in Kansas, USA (Lindsey et al.,
13 2001), and most of the publications were from the United States of America (Table 1). The
14 highest antibiotic concentrations were found in Asia: oxytetracycline has been found at 484
15 µg/L more than 20 km downstream from a WWTP in the Xiao river (Li et al., 2008).
16 Sulfamethazine has been reported with a concentration of 19 µg/L in Vietnam (Managaki et
17 al., 2007), and aus der Beek et al. (2016) reported ofloxacin and sulfamethoxazole at 17.7
18 µg/L and 14.3 µg/L respectively (Table 1). Finally, in Africa, sulfamethoxazole was reported
19 at 53.8 µg/L in Mozambique (Segura et al., 2015) and at 38.9 µg/L in Kenya (Madikizela et
20 al., 2017) (Table 1). In South African streams and rivers, nalidixic acid and ciprofloxacin
21 were reported with concentrations of 23 µg/L and 14 µg/L respectively (Agunbiade and
22 Moodley, 2016) (Table 1).

23 Segura et al. (2015) reported that the mean concentration of antibiotics in
24 contaminated fresh waters was significantly higher in low income countries, due to a lack of
25 sanitation facilities and wastewater treatment. We found similar results as the mean

1 concentration of antibiotics was 17.7 $\mu\text{g/L}$ and 11.3 $\mu\text{g/L}$ in Asia-Pacific and Africa
2 respectively, and 0.9 $\mu\text{g/L}$ and 0.4 $\mu\text{g/L}$ in America and Europe (for Europe: mean
3 concentration based on the reports in electronic supplementary material, table S1). It is worth
4 pointing out that consumption of antibiotics is higher in high-income countries (Klein et al.,
5 2018).

6 The studies paint a highly divergent picture, with different antibiotics found at vastly
7 different concentrations but overall concentrations found in surface waters are lower than
8 concentrations found in effluent wastewaters as pointed out in section 2. Furthermore, a
9 standardised panel for the determination of antimicrobial concentrations in the environment
10 has not yet been agreed on in these countries. Nevertheless, the presence of these
11 antimicrobials and their often-unquantified metabolites is of concern.

12

13 **4. Toxic potential of antibiotics**

14 While it is important to know about the presence and concentrations of antibiotics occurring
15 in fresh waters, it is arguably more important to determine whether the compounds have an
16 effect on the different organismal groups inhabiting the environment. Here, the challenge of
17 replicating the complex natural setting in the laboratory becomes apparent. For bacteria,
18 antibiotic susceptibility profiles are obtained under highly reproducible rich media
19 conditions, to eliminate variation between laboratories, because antibiotic susceptibility is
20 highly condition dependent (Greulich et al., 2015). It is questionable whether these profiles
21 can be readily applied to natural freshwater systems where nutrients are often limited.

22 To evaluate the antibacterial toxicity effects on non-target organisms, specific tests
23 measure either acute effects (often cell death or mortality rates, i.e. antibiotics short term
24 effects) or chronic toxicity (often exposure over a prolonged period of time, growth index or
25 reproduction, i.e. long term effects of antibiotics) (Soares et al., 2012). Multi-component

1 bioassays typically measure the overall response to a substance and assess the impact on
2 different levels of biological organisation such as community, population, individual and/or
3 sub-organism level (Carvalho et al., 2014). Bioassay measures are the EC₅₀ (concentration
4 giving half of the maximum response), LC₅₀ (concentration giving half lethality), IC₅₀
5 (concentration giving half inhibition), NOEC (concentration with no observed effect) and
6 LOEC (concentration giving the lowest observed effect). Such tests are generally regarded as
7 good indicators of the potential risks posed by individual chemicals, but there is no model
8 system and by design they fail to take into account chemical-chemical or chemical-
9 organismal interactions.

10 The database “Wikipharma” (Molander et al., 2009) compiles publicly available
11 ecotoxicity data for Active Pharmaceutical Ingredients (APIs), including antibiotics
12 (www.wikipharma.org, DOI: 10.1016/j.yrtph.2009.08.009). The rationale for choosing
13 “Wikipharma” as an example for a database was that it is a free, interactive, comprehensive
14 and up-to-date database on the effects caused by pharmaceuticals on non-target animals
15 (Molander et al., 2009). Our aim was not to perform a meta-analysis as such but to illustrate
16 that many antibiotics show high toxicity in bioassays with freshwater organisms (single
17 celled pro- and eukaryotes and multicellular organisms). We searched the database for studies
18 on toxicity tests using antibiotics and freshwater organisms and found 49 publications, which
19 - taken together - report the effects of 47 antibiotics in 513 different experiments. We define
20 one experiment as the measured effect of one antibiotic concentration on one species or on
21 bacterial communities (see the electronic supplementary material table S2). More than 50%
22 of these bioassay experiments included non-target bacteria, including 71 studies on
23 cyanobacteria. The antibiotics most studied in bioassays were trimethoprim (57 experiments
24 – 11%), sulfamethoxazole and oxytetracycline (48 – 9%), chloramphenicol (35 – 7%),
25 erythromycin (31 – 6%), ofloxacin (27 – 5%), and ciprofloxacin and ampicillin (25 -5%), the

1 remaining antibiotics were studied in less than 20 experiments (see the electronic
2 supplementary material table S2).

3 Most of the antibiotics used in ecotoxicological assays were found in fresh waters
4 (Fig. 1) and in most cases EC_{50} was reported. The Commission of the European Communities
5 classifies chemicals with an EC_{50} between 10 and 100 mg/L as harmful, from 1 to 10 mg/L as
6 toxic and those under 1 mg/L as very toxic to aquatic organisms (European Commission,
7 1996; Petrie et al., 2014) (Table 2). Interestingly, for unicellular organisms, the average EC_{50}
8 was below 1 mg/L (i.e. the antibiotics were ‘very toxic’) and in some cases the EC_{50} was
9 measured at much lower concentrations (Fig. 1A) and within the range found in streams and
10 rivers with antibiotic pollution (Table 1). Half of the studies that used concentrations below
11 10 μ g/L showed that these concentrations were harmful to single-celled pro- and eukaryotes.
12 For instance, some of these bioassay studies revealed that low concentrations (in the μ g/L
13 range) of antibiotics can have negative effects on bacteria (Table 2) and a third of the tests on
14 bacteria, looking at survival or bioluminescence (i.e. acute toxicity), measured EC_{50} below 1
15 mg/L or even in the microgram range. For example, Ando et al. (2007) tested the effects of
16 ampicillin on the cyanobacterium *Microcystis aeruginosa*, with a range of concentrations
17 from 30 ng/L to 200 mg/L, and measured EC_{50} at only 0.2 μ g/L (see Table 2).

18 According to the Wikipharma data, 25% of all studies estimating the effects of
19 antibiotics on eukaryotic, single celled algae, found EC_{50} values below 1 mg/L and 12 studies
20 even report EC_{50} to be below 100 μ g/L (electronic supplementary material table S2). The
21 tests performed were measured as growth inhibition, usually after 72 hours. Two studies, one
22 investigating the effects of amoxicillin on *Synechococcus leopolensis* and one of
23 clarithromycin on *Pseudokirchneriella subcapitata* showed very high sensitivity of
24 eukaryotic algae to antibiotics, as they found the EC_{50} to be 2 μ g/L (Andreozzi et al., 2004;
25 Isidori et al., 2005) (Table 2).

1 In the Wikipharma database, multicellular species generally showed EC₅₀ values
2 above 1 mg/L and all average values were in 10-1000 mg/L range (Fig. 1B). Therefore, they
3 seem less susceptible to antibiotics and are possibly not influenced by those concentrations
4 measured in running waters (Table 1) but the database showed notable exceptions, such as
5 duckweed. Multicellular plants used in bioassays were represented by duckweed and 14
6 experiments using this plant found an EC₅₀ for growth inhibition under 1 mg/ L (Brain et al.,
7 2004; Robinson et al., 2005) (Table 2).

8 Metazoans have also extensively been used in bioassays reported in Wikipharma
9 (Table 2), including species belonging to: fish (40 studies), molluscs (16), crustaceans (117),
10 rotifers (19) and cnidarians (4). Low levels of antibiotics directly in contact with invertebrates
11 (such as crustaceans, cnidarians and molluscs) did not affect their degree of survival, their
12 reproduction, nor their sex-ratio, with the exception of a few cases, and only five of the 137
13 experiments measured an EC₅₀ lower than 1 mg/L (Calleja et al., 1993; Isidori et al., 2005).

14 Therefore, the Wikipharma data base search on bioassays indicates that single-celled
15 organisms (both pro- and eukaryotes) are most susceptible to low antibiotic concentrations.
16 These findings are backed up by more studies and reviews. For example, Bengtsson-Palme
17 and Larsson (2016) reviewed the effects of antibiotics on clinically relevant bacteria and
18 found the lowest minimal inhibitory concentrations (MIC) at 2 µg/L for 13 antibiotics,
19 corresponding to the lowest concentration tested. Le Page et al. (2007) calculated relative
20 species sensitivity and found that prokaryotes were the most sensitive to antibiotics, and
21 Valitalo et al. (2017) who reviewed the toxicological impact of antibiotics on microscopic
22 aquatic organisms found that cyanobacteria and ammonium oxidizing bacteria were the most
23 sensitive to antibiotics in general. Therefore, natural microbial communities are the most
24 vulnerable to antibiotics discharge in the aquatic environment (as also pointed out by Grenni
25 et al. (2018)).

1 Taking this literature into account, and judging from the examples listed in the
2 database, we can identify some particularly problematic antibiotics in terms of their
3 occurrence in fresh waters and their toxicity. While some of the antibiotics affecting those
4 organisms are not frequently found in the environment, some others like ciprofloxacin and
5 ofloxacin can be found at relatively high concentrations in fresh waters (aus der Beek et al.,
6 2016; Feitosa-Felizzola and Chiron, 2009; Ginebreda et al., 2010) (Table 1) and are among
7 the most potent antibiotics at low concentrations (Bengtsson-Palme and Larsson, 2016).
8 Studies found that ciprofloxacin was inhibiting the growth of the cyanobacteria *Mycrocystis*
9 *aeruginosa* at 5 µg/L (Halling-Sørensen et al., 2000) and 17 µg/L (Robinson et al., 2005).
10 This antibiotic was found in fresh waters at concentrations in a similar range: 14.33 µg/L in
11 South Africa (Agunbiade and Moodley, 2016), and 9.66 µg/L in France (Feitosa-Felizzola
12 and Chiron, 2009) (Table 1). Ofloxacin can inhibit the growth of the bacterium *Vibrio*
13 *fischeri* at 0.9 µg/L and the algae *Pseudokirchnella subcapitata* at 4.74 µg/L (Table 2), and
14 was detected up to 17.7 µg/L in Asia (aus der Beek et al., 2016) and up to 8.7 µg/L in Spain
15 (Ginebreda et al., 2010). Similarly, chloramphenicol, erythromycin, norfloxacin,
16 oxytetracycline, streptomycin, and tylosin are classified as ‘very toxic’ for aquatic organisms
17 (European Commission, 1996; Petrie et al., 2014) and they have been detected at
18 concentrations of over 1 µg/L in fresh waters (Table 1).

19 Bioassays are useful to study the toxicity of new pharmaceutical compounds and the
20 EC₅₀ data give us some indication of the antibiotics’ toxicity in an environmental context,
21 however, they do not give information on the effect antibiotics can have in a complex natural
22 system. Also, they do not demonstrate that prokaryotes have the ability to metabolise
23 antibiotics and to evolve in response to antibiotic pollution.

24

25 **5. Antibiotic resistance in natural bacterial communities**

1 Standard bioassays do not provide information about microbial resistance, but we know that
2 environmental antimicrobials can act in that way. For instance, many studies have linked
3 antibiotic occurrence in wastewater and antibiotic resistance in WWTP impacted streams
4 (Guo et al., 2017; Schwartz, 2003). Significant correlations are found between the exposure
5 to antibiotics and their corresponding antibiotic resistance genes (ARGs), and the selective
6 pressure for ARGs increase with the concentrations of antibiotics (Li et al., 2012; Rodriguez-
7 Mozaz et al., 2015). It is known that very low antibiotic concentrations (10–100 times below
8 clinically determined minimum inhibitory concentrations) can increase the relative
9 abundance of resistant bacteria and select for resistance by promoting the rate of adaptive
10 evolution (Friman et al., 2015; Gullberg et al., 2011; Lundström et al., 2016).

11 The emergence of resistance to antimicrobial compounds such as antibiotics is a
12 natural process that has its origins, much as many antibiotics themselves, in inter-species
13 competition in natural environments. It is not surprising, therefore, that bacteria resistant to
14 antibiotics have been found in relatively pristine aquatic and soil environments. A seminal
15 study by Dantas et al. (2008) showed that communities of soil bacteria were able not just to
16 tolerate antibiotics, but to use them as their only source of carbon.

17 In the clinical context, near consensus has been reached that overuse of available
18 antibiotics combined with the failure to discover new ones is likely to be one of the key
19 challenges of the healthcare sector in the 21st century (Piddock, 2012). Most research has,
20 understandably, focussed on this issue, elucidating genetic determinants of secondary
21 resistance, i.e. mutations that confer increased tolerance to the antibiotic upon exposure to it.
22 This differs from primary or intrinsic resistance that can have a number of reasons, for
23 example the inability of most penicillins to reach their target site in Gram-negative bacteria.
24 Mechanisms of resistance, e.g. degradation or modification of the antibiotic, upregulation of
25 efflux pumps, modification of the target enzyme/substructure rendering the antibiotic useless

1 and ways for genetic dissemination between bacteria via horizontal gene transfer (HGT) are
2 well understood (Blair et al., 2014).

3 While the potential for wide-spread resistance can therefore be regarded as near
4 ubiquitous, the dynamics promoting and limiting the spread of resistance across bacterial
5 populations in the natural environment, both within and between species are less well
6 understood. Importantly, the effect of sublethal levels of antibiotics and resulting
7 physiological and phenotypic adaptations, which are governed by environmental factors and
8 also greatly influence (future) resistance potential have recently gained increased attention
9 (Dörries et al., 2014; Fernández et al., 2011).

10 It is important to determine dynamics promoting and limiting the spread of resistance
11 across bacterial populations in the natural environment, and to identify the environments
12 where an increase of ARGs can occur. Chemicals driving resistance to antibiotics can include
13 – in addition to antibiotics themselves – biocides such as disinfectants, metals and
14 phytochemicals (Friedman, 2015; Gullberg et al., 2014; Pal et al., 2015; Wales and Davies,
15 2015). Moreover, new research is showing that microplastic can be a vehicle for antibiotics
16 and resistance genes in aquatic ecosystems (Arias-Andres et al., 2018; McCormick et al.,
17 2014). In contrast with other chemical pollutants, bacterial contaminants and their ARGs are
18 capable of persisting and spreading in the environment, and ultimately could transfer their
19 resistance to human pathogens (Bengtsson-Palme et al., 2018; Berendonk et al., 2015).
20 Integrating the capacity of ARGs to transfer from contaminated sources to pathogenic
21 bacteria into risk assessment is a considerable challenge and we also need to be able to test
22 for the occurrence of resistance determinants in the environment (Berendonk et al., 2015).

23 Several studies are looking into the effects of sub-inhibitory concentrations of
24 antibiotics in complex aquatic bacterial communities. Quinlan et al. (2011) exposed a stream
25 biotic community to tetracyclines (0.5-100 µg/L) and found changes in antibiotic resistance,

1 bacteria abundance and productivity after seven days. Lundstrom et al. (2016) also tested the
2 effects of tetracyclines on biofilms (0.1-1000 µg/L) and found an increase in antibiotic
3 resistance bacteria at 10 µg/L and changes in the biofilm taxonomic composition. The
4 development of antibiotic resistance in natural microbial communities is an indirect effect of
5 antibiotics in the environment but they can also have direct effects on the microbial
6 community structure and functioning. Bactericides can cause the disappearance of microbial
7 populations and change the entire bacterial community. Proia et al. (2013) found alterations
8 in the bacterial community composition when exposed to a mixture of 16 antibiotics
9 (concentrations ranging from 0.005 to 1.5 µg/L) with an increase in Actinobacteria and
10 Laverman et al. (2015) found an effect on bacterial community structures when exposed to
11 vancomycin (1000 µg/L) in river sediments downstream of a WWTP.

12 Low concentrations of antibiotics can also play an important role in the structure of
13 biofilms, for example, low concentrations of the antibiotic tobramycin (50-1000 µg/L)
14 induced biofilm formation in *Pseudomonas aeruginosa* by modulating the aminoglycoside
15 resistance gene which also regulates cell surface adhesiveness (Hoffman et al., 2005). The
16 effect of sub-lethal levels of antibiotics and resulting physiological and phenotypic
17 adaptations, which are governed by environmental factors, have recently gained increased
18 attention (Dorries et al., 2014; Fernández et al., 2011). Furthermore, physiological and
19 genetic adaptations to antibiotic stress do not happen in isolation, but are often tied to
20 changes in other bacterial behaviours such as virulence (Behrends et al., 2013; Martínez and
21 Rojo, 2011; Watkinson et al., 2007; Yeung et al., 2011).

22

23 **6. Evaluation of antibiotics under more complex and realistic scenarios**

24 Our literature search shows that antibiotic concentrations measured in fresh waters, despite
25 concentrations being well below clinically-relevant levels, are very likely to have direct, and

1 indirect, effects on at least the microbial component of freshwater communities.
2 Environmental scientists are now challenged to show if antibiotic loadings can be detrimental
3 for freshwater communities - as a mixture and under global change scenarios. To capture the
4 true effects of antibiotic impacts on whole communities, and the ecosystem processes they
5 drive, we propose three major future research avenues: i) food webs, ii) antibiotic mixtures
6 and iii) interaction with other stressors such as temperature (Fig. 2), as we outline in the
7 following paragraphs.

8 The impact of antibiotic pollution on bacteria and aquatic food-webs is likely to be
9 substantial but is complex to determine. While standard bioassays are a valid tool to elucidate
10 the effects of one contaminant on one single species, they generally do not provide
11 information as to how antibiotic exposure influences species assemblages and therefore
12 ecosystem functioning. Sub-inhibitory concentrations of antibiotics can have potential effects
13 on species interactions such as changes in population dynamics and bacterial community
14 composition which can lead to changes in trophic interactions but we are still lacking a
15 population-level perspective (Grenni et al., 2018; Hiltunen et al., 2017). A recent study also
16 found that cooperative communities (i.e. when metabolites produced by one organism are
17 used as nutrients or energy source by another) are more susceptible to antibiotics and as
18 cross-feeding is nearly ubiquitous in microbial communities, low antibiotic concentrations
19 might have a stronger effect than previously thought (Adamowicz et al., 2018).

20 In addition to bacteria interacting with other bacteria, important interactions in natural
21 microbial communities are those with protozoan predators. Protozoans are the most abundant
22 predators feeding on bacteria and can have a profound effect on bacterial abundance. There
23 are experiments showing that protozoan predators and antibiotic resistance are linked. One
24 study shows that antibiotics can lead to cascading indirect effects in species in other trophic
25 levels (Friman et al., 2015). This study used the bacterium *Pseudomonas fluorescens* and its

1 protozoan consumers (the ciliate *Tetrahymena pyriformis* and the flagellate *Chilomonas*
2 *paramecium*) to explore the effects of 40 µg/L of gentamicin and found that bacteria
3 increased resistance to both antibiotics and predators in a manner dependent on the antibiotic
4 concentration (Friman et al., 2015). The bacterial adaptation cascaded through the food web
5 leading to a reduced predator-prey abundance ratio, which increased the instability of
6 populations and lowered the predator community (Friman et al., 2015). In another study the
7 protozoan predator *Tetrahymena termophila* increased the persistence and spread of an
8 antibiotic resistance plasmid in populations of the bacterial pathogen *Serratia marcescens*
9 (Cairns et al., 2016).

10 In other food web studies including metazoan consumers, Quinlan *et al.* (2011)
11 looked at the effect of tetracycline in stream mesocosms and observed a decrease in bacterial
12 abundance but also a reduction in the algal periphyton biomass and nematode abundance.
13 Additionally, they showed that at the highest dose of tetracycline (10 µg/L and 100 µg/L),
14 and after 28 days of non-exposure, the bacterial productivity recovered but not the abundance
15 of bacteria, algae and nematodes (Quinlan et al., 2011). Little is known about the extent that
16 antibiotics are taken up and redistributed among different types of organisms within a food
17 web. A recent study investigated the bioconcentration of trimethoprim over several months in
18 an aquatic food web but they did not detect the antibiotic in the biota (Lagesson et al.,
19 2016). Simple laboratory experiments could explore whether antibiotics change the
20 composition of microscopically small aquatic communities - consisting of bacteria, viruses
21 and protists (Fig. 2; e.g. following the approach used by Friman et al. (2015)). Laboratory
22 cultures could even include larger invertebrates (such as microscopically small crustaceans)
23 to explore how changes in the microbial food web influence the reproduction of larger
24 invertebrates. Certainly it is important to expand these experiments to the 'real world' and
25 natural biofilms are an ideal system to test the effects of antibiotics (Lundström et al., 2016;

1 Quinlan et al., 2011). We believe an important focus is predator-prey oscillations with prey
2 that is evolving resistance (see Fussman et al., 2014 for an example of a microcosm set-up
3 and data analysis).

4 Another complex angle to antibiotic pollution in the environment is that organisms
5 and food webs are faced with ‘antibiotic cocktails’. Different classes of antibiotics are
6 typically detected simultaneously in fresh water (Table 1) and therefore, aquatic organisms
7 are exposed to mixtures of antibiotics. The individual concentrations of antibiotics that are
8 measured in the environment might be low, but the combined concentrations could result in
9 significant toxicity to aquatic organisms. For example, in the Llobregat River basin in Spain,
10 four antibiotics were analysed (erythromycin, sulfamethoxazole, trimethoprim and ofloxacin)
11 with very low individual concentrations but with a total concentration of 2.4 µg/L (López-
12 Roldán et al., 2010).

13 Chemicals in mixtures potentially interact with each other, in synergy or antagonistically.
14 It is therefore essential to investigate the potential interactions between antibiotics in the
15 environment but only a few studies have done this. A theoretical link between the toxicity of
16 the individual antibiotics and the effects of a mixture would allow the prediction of the
17 effects of antibiotic mixtures on aquatic organisms. Two principle concepts, the concentration
18 addition (CA) and the independent action (IA), are often applied for the assessment of
19 pharmaceutical mixtures in the environment (Backhaus, 2014).

20 For example, the mixture toxicity of ten quinolones (from 14 µg/L to 1020 µg/L) on the
21 test organism *Vibrio fischeri*, was estimated using these two concepts and results indicated
22 that the mixture was best predicted with the concentration addition (Backhaus et al., 2000).
23 González-Pleiter et al. (2013) also showed that the combined effect can be ‘more than the
24 sum of its parts’ by testing the effects of antibiotics used singly, binary and in mixtures of
25 four and five antibiotics. The toxicological interactions of the antibiotics were analysed using

1 the combination index (CI) and they showed that the combination of erythromycin and
2 tetracycline had a synergistic effect on the green algae *Pseudokirchneriella subcapitata* at
3 environmentally relevant concentrations (González-Pleiter et al., 2013). Erythromycin and
4 tetracycline added together inhibited the growth of the algae at only 1/8th of their individual
5 inhibition concentrations. However, to date, synergistic or antagonistic effects have rarely
6 been observed in multi-components mixtures.

7 The individual concentrations of antibiotics, which are measured in the environment,
8 can be low but the combined concentrations could result in significant toxicity for aquatic
9 organisms (Fig. 2). An example for a study that attempted a more complex approach comes
10 from Bundschuh et al. (2009), who tested the effects of antibiotic mixtures with
11 sulfamethoxazole, trimethoprim, erythromycin, roxithromycin and clarithromycin on a
12 decomposer detritivore system (the freshwater crustacean *Gammarus fossarum* feeding on
13 leaves colonised by bacteria and fungi). They found that fungal biomass was higher at a total
14 antibiotic concentration of 200 µg/L and thus *Gammarus* preferred leaves ‘contaminated’
15 with this antibiotic cocktail (Bundschuh et al., 2009). It was suggested that the antibiotic
16 mixture inhibited bacterial growth, which reduced competition for fungi and therefore
17 promoted fungal growth (Bundschuh et al., 2009) but the design of the experiment did not
18 allow mechanisms to be extracted.

19 It is essential to investigate the potential interactions between antibiotics in the
20 environment. These questions could be addressed in experiments that mirror the logic of
21 biodiversity-ecosystem functioning research, where the effects of single vs. multi-species
22 treatments on ecosystem processes have been assessed (Lawrence et al., 2012; Perkins et al.,
23 2015; Reiss et al., 2011, 2010, 2009). In analogy to these experiments, it is possible to run
24 laboratory experiments with antibiotic mixtures and to investigate their single- and mixture
25 effects, both on individual species (bacteria) or mini food webs. As a response variable, we

1 consider the adaptation of bacteria to antibiotic mixtures to be a priority research focus as
2 adaptation to antibiotics can induce changes in the bacterial community and bacteria
3 consumers can be susceptible to them.

4 In ecosystems, the effects of antibiotics are complex and context-dependent, as
5 highlighted by taking food webs and mixtures into account, and environmental scientists are
6 faced with the fact that antibiotics are not the only stressors in the system. The rise of
7 ‘ecological surprises’ in the primary scientific literature highlights the growing uncertainty
8 over the cumulative impacts of multiple novel and extreme environmental changes, or
9 ‘stressors’ (Jackson et al., 2016). Ecological surprises are unexpected findings on the
10 combined effects of stressors such as synergism or antagonism. The organisms in the
11 environment are not only exposed to climate change or land use or pollution alone but
12 simultaneously to a multitude of stressors (Fig. 2). If those other stressors have an impact on
13 the diversity of microbial communities, it could affect the spread of antibiotic resistance
14 genes via HGT as species composition and diversity can be a major factor determining how
15 resistance genes spread (Tamminen et al., 2012). For example, a study shows that plasmid
16 transfer is likely to occur to abundant strains and to low-abundance strains in the presence of
17 a more structured spatial environment (Cairns et al., 2018). This study also suggests that
18 plasmid transfer is more likely to happen to more bacterial strains if antibiotics are present
19 (Cairns et al., 2018).

20 Therefore, it is important to consider how other factors, such as temperature, change
21 the effects of antibiotics. Temperature is a key factor to include in antibiotic studies because
22 both chemical reactions, and the metabolic activity of freshwater organisms, are governed by
23 strict physical laws. In aquatic ecology, theoretical frameworks that include temperature and
24 traits of organisms already exist and can explain how communities respond to temperature
25 changes (Perkins et al., 2015). The use of microcosms, mesocosms and field experiments

1 have improved our ability to predict effects of contaminants on higher levels of biological
2 organisation (Thompson et al., 2015) as we can test complex, multifactorial hypotheses
3 concerning interactions between the contaminants and environmental factors.

4

5 **7. Concluding remarks**

6 In this paper, we illustrate that antibiotic pollution in fresh water is ubiquitous and that
7 concentrations in the field are substantial; that many antibiotics are toxic for fresh water
8 organisms from bacteria to multicellular organisms; and that even sub-lethal concentrations
9 have the ability to induce changes in freshwater communities via bacterial resistance. This
10 has knock-on effects for species interactions within the entire fresh water community and is
11 complicated by the fact that antibiotics interact with each other and other stressors. Clearly,
12 freshwater ecologists have the tools to show how antibiotic pollution has, and will, impact
13 upon natural freshwater communities. We believe that it is possible to run experiments that
14 can build on recent research (such as protozoans grazing on adapted bacteria) to find out
15 under which scenarios antibiotics affect natural communities (e.g. competition between
16 prokaryotes and fungi) and how antibiotic effects can cascade from prokaryotes to the entire
17 ecosystem.

18 It is obvious that antibiotic mixtures can have an effect on important ecosystem
19 processes such as organic matter decomposition in stream ecosystems that are also
20 experiencing stressors such as species loss, habitat alteration and warming. Further, we
21 should also keep in mind that antibiotics are not the only substances found in the fresh water
22 and that complex chemical mixtures with other pollutants should be tested under
23 environmentally relevant scenarios.

24 Going forward, we believe some general considerations can help future studies.

25 Firstly, in a food web context, we have to consider predator-prey oscillations with prey that is

1 evolving resistance. Secondly, in the field, the combined concentration of antibiotics can be
2 high but whether effects are synergistic, additive or antagonistic depends on the particular
3 antibiotics. Therefore, it is critical to remember that for mixtures of antibiotics the whole
4 effect might be different from the sum of its parts. Thirdly, it is essential to move beyond a
5 bioassay approach and to consider the context-dependency of sub-lethal antibiotic
6 concentrations (i.e. realistic field concentrations), to assess fluctuations within the microbial
7 food web through time and to estimate how interactions change with temperature and other
8 anthropogenic stressors.

9

10 **Data accessibility**

11 The datasets supporting this article have been uploaded as part of the supplementary material.

12 **Competing interests**

13 We declare no competing interests.

14 **Authors' contributions**

15 M-CD carried out the literature search, data compilation, and wrote the manuscript; JR, VB
16 and AR wrote the manuscript; M-CD and JR conceived of the study. All authors gave final
17 approval for publication.

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1 **Figure legends**

2 **Fig. 1.** Ecotoxicity (EC₅₀) of antibiotics, based on bioassays published in the database
3 Wikipharma, for unicellular organisms (panel A) and for multicellular organisms (panel B).
4 The responses measured were both chronic (e.g. growth inhibition, bioluminescence) and
5 acute (e.g. death, survival) and are given in the electronic supplementary material Table S2.
6 Only antibiotics used in at least 3 bioassays are represented. Boxes show interquartile range
7 and median, whiskers show range and outliers. The widths of the boxes vary with the number
8 of experiments (n). The red lines indicate the toxicity level: the Commission of the European
9 Communities classifies chemicals with an EC₅₀ between 10 and 100 mg/L as harmful, from 1
10 to 10 mg/L as toxic and those under 1 mg/L as very toxic to aquatic organisms (European
11 Commission, 1996; Petrie et al., 2014). The black triangles represent the highest
12 concentrations of the antibiotic as found in fresh water (see Table 1).

13

14 **Fig. 2.** Possible effects of antibiotics and multiple stressors in freshwater ecosystems on a
15 simple freshwater food web (bacteria, algae, a protist and a micro-crustacean). Arrows
16 indicate trophic links between species (thin arrow: normal prey-to-predator ratio, thick arrow:
17 destabilised prey-to-predator ratio). Left hand side panel: the food web without external
18 stressors. Middle panel: the effect of a single antibiotic that might induce the death of one
19 strain of bacteria and the resistance of another one, impacting the whole food web by
20 changing the trophic links. The right-hand side panels show three further scenarios when
21 more stressors are added such as: other antibiotics (the effect of a cocktail of antibiotics
22 might induce stronger and additive effects on both bacteria and algae), or warming (the effect
23 of antibiotics coupled with the effect of warming might induce an increase of the organisms'
24 metabolic rates and therefore a quicker resistance for some bacteria), or warming and other
25 antibiotics together.