The Influence of pH and Dissolved Organic Carbon on the Ecotoxicity of Ampicillin and Clarithromycin

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ABSTRACT

The impacts of water chemistry properties including pH and dissolved organic carbon (DOC) on the ecotoxicity of active pharmaceutical ingredients (APIs) are increasingly evident. These impacts are a result of alterations in API bioavailability: pH regulates the bioavailability of many ionizable APIs via chemical speciation, whereas DOC interacts with several APIs to inhibit the APIs from traversing the membrane system of organisms. In this study, we examined the influences of pH and DOC on the bioavailability of ampicillin (AMP) and clarithromycin (CLA) with the help of a bioavailability model. The effects on bioavailability were quantified by ecotoxicity observed in cyanobacteria growth inhibition tests with *Microcystis aeruginosa PCC7806*. The median effect concentration (96h-$EC50_{total}$) of AMP increased by 5-fold when pH raised from 7.4 to 9.0, suggesting the zwitterionic $AMP^{+/-}$ species being higher in bioavailability than the negatively charged AMP^{-} species. CLA ecotoxicity showed no significant pH-dependency, suggesting CLA^{+} and CLA^{0} species to be equally bioavailable, albeit it correlated significantly with *M. aeruginosa* growth rate in negative controls. In addition, DOC demonstrated no significant effects on the ecotoxicity of AMP or CLA. Overall, together with earlier results on ciprofloxacin, our data show that bioavailability relations with pH and DOC are variable among different antibiotics. Factors other than chemical speciation alone could play a role in their bioavailability, such as their molecular size and polarity.

KEYWORDS

Bioavailability, antibiotics, ecotoxicology, ionizable compound.

ABBREVIATIONS

AMP, ampicillin; AMR, antimicrobial resistance; APIs, active pharmaceutical ingredients; CLA, clarithromycin; DOC, dissolved organic carbon; EDTA, ethylenediaminetetraacetic acid; ERY, erythromycin; MoA, mode of action; PCC, Pasteur Culture Collection of Cyanobacteria; PPCPs, pharmaceutical and personal care products;

SPE, Solid phase extraction; WC, Wright Chu.

1. INTRODUCTION

The occurrence of active pharmaceutical ingredients (APIs) in surface water has raised concerns about their potential adverse effects on aquatic organisms and the spread of antimicrobial resistance (AMR)[1]. In water bodies, the ecotoxicity of an API is associated not only with its occurrence, e.g., total measured concentration, but also with its bioavailability. Water chemistry factors such as pH, water hardness, and dissolved organic carbon (DOC) are well-known regulators of the bioavailability of heavy metals[2]. Recently, the influence of environmental factors on the bioavailability of APIs has also become increasingly evident. pH-dependent toxic effects were reported for three antihistamines dimethindene, doxylamine, ketotifen, on zebrafish [3]. Water browning, which is related to concentrations of light-absorbing DOC compounds, was also found to increase the tolerance of a microalgae population to a mixture of 12 pharmaceutical and personal care products (PPCPs)[4].

The influence of pH on bioavailability is often linked to its effects on the chemical speciation of ionizable compounds. Ionizable chemicals are estimated to account for > 60% of pharmaceutical drugs[5,6]. Consequently, local pH conditions of natural waterbodies can determine the ionization status of many APIs, which might affect how the API molecules traverse the membrane system of aquatic organisms. The three major pathways for transmembrane transport, i.e., passive diffusion, facilitated diffusion, and active transport, differ in their energy demand[7]. Amongst them, passive diffusion allows chemicals to penetrate membranes following a concentration gradient without energy support (necessary for active transport) or carriers' mediation (necessary for facilitated diffusion). According to the pH-partition hypothesis[8], APIs in the neutral or zwitterionic states are more likely to take advantage of passive diffusion than APIs in charged states. As a result, the molecules carrying a zero net-charge might have a higher bioavailability and consequently higher ecotoxicity than their charged counterparts. This phenomenon has been observed in several studies, e.g., for cetirizine and ketotifen to *Danio rerio* embryo[3], for sulfadiazine to *Daphnia magna*[9], for propranolol to *Scenedesmus vacuolatus*[11], and for ciprofloxacin to *Microcystis aeruginosa*[11].

DOC are mainly reported to reduce the bioavailability of organic pollutants. The molecules that bind to DOC become less capable of traveling across the cell wall and cell membrane than their freely dissolved counterparts, because organic pollutant-DOC complexes are too large in terms of size[12,13]. Thus, the ecotoxicity of these organic pollutants decreases in the presence of DOC. The interaction between very hydrophobic organic substances (HOS) (i.e., octanol-water partitioning coefficient $K_{ow} > 10^5$) and DOC is predictable using the hydrophobicity of chemicals, because the HOS-DOC interaction is driven by solvophobic partitioning[14,15]. For less hydrophobic chemicals including many APIs, however, their interactions with DOC are more complicated and affected by multiple mechanisms, such as cation exchange, van der Waals forces, electrostatic interactions, H-bonding, and complexation[16,17]. Thus, API-DOC interactions are more challenging to predict, because the interactions are affected by the physical-chemical properties of the APIs (i.e., K_{ow}, molecular structure), the characteristics of the DOC (i.e., composition, molecular size), and the ionizing state of both the APIs and DOC[16,18,19].

In this study, we investigated the influence of pH and DOC on the bioavailability of two relatively hydrophilic antibiotic pharmaceuticals, i.e., ampicillin (AMP) and clarithromycin (CLA) (Figure S7). Our goal was to examine whether the effects of water chemistry, as earlier identified for ciprofloxacin[11], can be generalized towards other antibiotics. The two compounds are associated with the spread of AMR resistance in aquatic environments[19, 20] but have distinct physical-chemical properties and mode of action (MoA). AMP is a β-lactam penicillin. It inhibits cell wall synthesis and activates endogenous autolytic mechanisms[21,22]. CLA is a macrolide that is rapidly biotransformed into a microbiologically active metabolite and binds reversibly to the 50S ribosomal subunit of bacteria, eventually inhibiting protein synthesis[23], although it is not efficient against gram-negative bacteria[24].

The ecotoxicity of AMP and CLA was examined in a range of pH (7.3-9.3) and DOC (0-20 mg L^{-1}) conditions using cyanobacteria growth inhibition experiments. The speciation of the two test compounds demonstrates opposite trends in the tested pH range: with increasing pH, $AMP^{+/-}$ gradually becomes negatively charged AMP^-, whereas the neutral CLA^0 overtakes the positively charged CLA^+ to become the dominant species Figure S1). Based on our hypothesis that the

92 zwitterionic $AMP^{+/-}$ and the neutral CLA^{0} are more bioavailable than the charged AMP^{-} and CLA^{+},
93 we expected high ecotoxicity of AMP and CLA to appear at respectively the lower and the higher
94 end of the tested pH range. The second hypothesis was that DOC reduces the bioavailability of
95 both AMP and CLA via binding. Bioavailability models modified from a ciprofloxacin
96 bioavailability model[11] were employed to facilitate the data analysis.

97 **2. MATERIALS AND METHODS**

98 2.1 Toxicity test

99 2.1.1 Test chemicals

100 Ampicillin hydrochloride ($C_{16}H_{19}N_3O_4S{\cdot}HCl$, CAS 69-52-3, pharmaceutical EU standard
101 grade) and clarithromycin ($C_{38}H_{69}NO_{13}$, CAS 81103-11-9, pharmaceutical EU standard grade)
102 were purchased from Sigma-Aldrich (Germancy). Stock solutions of AMP (50 mg L^{-1}) and CLA
103 (50 mg L^{-1}) were prepared with deionized water immediately prior to toxicity tests. The DOC
104 employed in the tests was Suwannee River natural organic matter (SRNOM, 2R101N), purchased
105 from the International Humic Substances Society. The DOC stock (318.52 mg L^{-1}) was prepared
106 in Milli-Q water and preserved at 4 °C.

107 2.1.2 Test organism and culture conditions

108 The freshwater cyanobacterium *Microcystis aeruginosa PCC7806* was obtained from the
109 Pasteur Culture Collection of Cyanobacteria (PCC). *M. aeruginosa* was cultured in Wright Chu
110 (WC) medium[25] at 24 ± 1 °C with continuous illumination of 27.0 ± 2.7 μmol m^{-2} s^{-1}. It is
111 assumed that ethylenediaminetetraacetic acid (EDTA) in the WC culture medium has no influence
112 on the bioavailability of test chemicals.

113 2.1.3 Cyanobacteria growth inhibition test

114 The cyanobacteria growth inhibition tests were adapted from the OECD guideline 201[26] and
115 were performed under the same conditions as the culture conditions described above. A
116 modification was made to the duration of the toxicity test, which was extended from 72 hours to

96 hours. Inoculum cultures were prepared 4 days before the toxicity tests. The initial cell density of *M. aeruginosa* employed in the tests was 40,000 cell mL^{-1}. This cell density level was chosen to ensure that *M. aeruginosa* was maintained in the exponential growth phase throughout the entire test period. During the test, cell density was measured every 24 hours by Beckmann Coulter Counter. The pH of the test cultures was adjusted manually twice a day by adding small volumes (10-30 μL) of sodium hydroxide solutions (0.1 M, 0.5 M) and hydrochloric acid solutions (0.1 M, 0.5 M) to 50 mL of the test medium. No pH buffers were applied. The *M. aeruginosa* growth rate was calculated from the measured cell densities and employed as the endpoint. For the calculation of the cell growth rate in a treatment, cell density measurements were transformed using the natural-logarithm and plotted against time. The growth rate was defined as the slope of the regression line.

2.1.4 Experimental design

Four experiments were performed in this study. Exp I and II used AMP as the test chemical. In Exp I, pH was the independent variable (nominal pH = 7.4, 7.9, 8.4, 9.3), whereas both pH (nominal pH = 7.3, 9.3) and DOC (nominal DOC = 0, 10, 20 mg L^{-1}) were independent variables in experiment II (full-factorial). Exp III and IV tested the toxicity of CLA. Exp III tested 5 pH levels (nominal pH = 7.2, 7.6, 8.1, 8.6, 9.3). Exp IV employed both pH (nominal pH = 7.3, 8.3, 9.3) and DOC (nominal DOC = 0, 10, 20 mg L^{-1}) as independent variables (full factorial). The pH and DOC concentration levels were selected to represent the common condition (90%) of natural waterbodies in Europe reported by the Forum of European Geological Surveys[27].

In the toxicity experiment, each test medium was subdivided into 6 groups, including 1 control group (with 6 replicates) and 5 treatment groups (with 3 replicates). The control groups contained no test chemical. The nominal concentrations of test chemicals in the 5 treatment groups were arranged in a geometric series with a spacing factor of 2. In Exp I and II, the nominal concentrations of AMP in experiment were 1.1-18.1 μg L^{-1} at pH 7.3 and 7.4, 2.3-36.2 μg L^{-1} at pH 7.9, and 4.5-72.4 μg L^{-1} at pH 8.4 and 9.3 (Table S1). In Exp III, the nominal concentrations

of CLA were 1.5-24.0 μg L^{-1} at pH 7.3 and 7.5, 0.8-12.0 μg L^{-1} at pH 8.0, 8.5, and 9.3. In Exp IV, the nominal concentrations of CLA were 0.8-12.0 μg L^{-1} at pH 7.3, 8.3, and 9.3.

In addition, a light exposure experiment (Exp V) was performed with AMP to study the degradation of AMP under the light condition employed by the toxicity test. AMP was dissolved in four types of modified WC media: A (nominal pH =7.4, no DOC, nominal AMP = 15 μg L^{-1}), B (nominal pH =7.4, nominal DOC = 20 mg L^{-1}, nominal AMP = 15 μg L^{-1}), C (nominal pH =9.3, no DOC, nominal AMP = 50 μg L^{-1}), D (pH =9.3, nominal DOC = 20 mg L^{-1}, nominal AMP = 50 μg L^{-1}). The four AMP solutions were each divided into three aliquots and placed for 96 hours on the same lightbox as the one where toxicity experiments had been conducted.

2.2 Instrumental analysis

From Exp I-IV, samples were collected at the beginning and at the end of the tests to measure concentrations of the test antibiotics, DOC and Ca^{2+}. The samples were filtered with 0.45 μm polyethersulfone (PES) membrane syringe filters. According to our preliminary test, the impact of the PES membrane filters on the concentration of AMP and CLA was below 5%. AMP and CLA samples were stored at -20 °C immediately after collection. From Exp V, AMP samples were collected at 0-, 24-, 48-, 72- and 96-hours of the test. DOC samples and acidified Ca^{2+} samples (1% formic acid) were stored at 4 °C. DOC samples were analyzed with a Total Organic Carbon Analyzer (Shimadzu TOC-L). The measured DOC concentration was corrected for the influence of EDTA present in the WC medium, by subtracting the background DOC concentration in the WC medium (2 mg L^{-1}). Ca^{2+} samples were analyzed with Inductively Coupled Plasma Mass Spectrometry (ICAP 7000 Series).

Samples for AMP and CLA measurements were processed and analyzed as described by Zhang et al[11]. Briefly, 3.0 mL samples were first added with 120 μL 25.0 g L^{-1} $Na_2EDTA.2H_2O$ solution, before the pH of the samples was adjusted to 3.0 ± 0.1 by the addition of 42 μL 0.5 M HCl. Solid phase extraction (SPE) of the modified samples was performed on Oasis HLB cartridges (6 mL, 200 mg sorbent). The cartridges were first conditioned with 6.0 mL HPLC-grade MeOH and 6.0 mL HPLC-grade H_2O and then loaded with 3.0 mL samples. Cartridges

were each washed with 18.0 mL HPLC-grade H_2O and eluted with 5.0 mL MeOH/ACN (50/50, v/v %). Extracts were dried with a gentle nitrogen flux and dissolved again in 1.0 mL reconstitute solvent (methanol/HPLC-grade water = 10/90, v/v %, containing 0.1% (v/v%) formic acid and 0.1 g L^{-1} $Na_2EDTA{\cdot}2H_2O$). Instrumental analysis was performed on a reversed phase ultra-high performance liquid chromatography system hyphenated with a Q-Exactive™ high-resolution Orbitrap mass spectrometer (Thermo Fisher Scientific). The calibration and quantification processes of the test chemicals are described in Section S4 in supplementary file.

2.3 Bioavailability model

2.3.1 Model structure

In the bioavailability models, the different species of a test chemical, i.e., $AMP^{+/-}$ and AMP^{-}, CLA^{+} and CLA^{0}, were assumed to differ in their individual toxicity to reflect the differences in bioavailability. The total toxicity, being the result of toxic effects caused by both charged and non-charged species, was calculated using the CA concept. The following equations use AMP as an example to show the structure of the used bioavailability model. The corresponding equations for CLA (Eq. S1-S4) are listed in Section S1 in supplemental file. First, the chemical speciation of AMP is simulated by the Henderson–Hasselbalch equation.

$$\log_{10}\frac{[AMP^{+/-}]}{[AMP^{+}]} = pK_{AMP,a1} - pH \quad \text{Eq. 1}$$

$$\log_{10}\frac{[AMP^{-}]}{[AMP^{+/-}]} = pK_{AMP,a2} - pH \quad \text{Eq. 2}$$

where $pK_{AMP,a1}$ and $pK_{AMP,a2}$ are the 1st (2.55) and 2nd (7.14) acid dissociation constant of AMP. Chemical speciation is assumed to reach an immediate equilibrium in the aqueous phase.

Based on the second hypothesis, DOC-AMP interaction is simulated as a binding process (Eq. 3), which quantifies the strength of the binding using a binding potency K_{AMP}^{x} (x = +/-, -).

$$[DOC-AMP] = [DOC]\ x\ (K_{AMP}^{+}\ x\ [AMP^{+}] + K_{AMP}^{+/-}\ x\ [AMP^{+/-}] + K_{AMP}^{-}\ x\ [AMP^{-}]) \quad \text{Eq. 3}$$

where [DOC–AMP] is the concentration of AMP bound to DOC (mol L^{-1}); [AMP^+], [$AMP^{+/-}$] and [AMP^-] are the aqueous concentrations of AMP^+, $AMP^{+/-}$ and AMP^- (mol L^{-1}), respectively; [DOC] is the concentration of DOC (g L^{-1}); and K_{AMP^+}, $K_{AMP^{+/-}}$, and K_{AMP^-} are the binding potency (L g^{-1}) between AMP^+ and DOC, between $AMP^{+/-}$ and DOC, and between AMP^- and DOC, respectively.

The toxic potency of the different species of AMP is described by their individual median effect concentration, e.g., EC50(AMP^+), EC50($AMP^{+/-}$), EC50(AMP^-). The overall toxicity ($EC50_{total}$) of the test chemical (referring to the total concentration of all AMP species together) is calculated using the concentration additive (CA) concept[11] (Eq. 4).

$$\frac{1}{EC50_{total}(AMP)} = \frac{fAMP^+}{EC50(AMP^+)} + \frac{fAMP^{+/-}}{EC50(AMP^{+/-})} + \frac{fAMP^-}{EC50(AMP^-)} \quad \text{Eq. 4}$$

where $EC50_{total}$ is the total median effect concentration in a specific environment (i.e., the sum of concentrations of all AMP species at 50% effect), $fAMP^+ = [AMP^+]/[AMP_{total}]$, $fAMP^- = [AMP^-]/[AMP_{total}]$, $fAMP^{+/-} = [AMP^{+/-}]/[AMP_{total}]$, EC50($AMP^+$), EC50($AMP^{+/-}$) and EC50($AMP^-$) are the median effect concentrations of AMP^+, $AMP^{+/-}$ and AMP^-, respectively. The AMP molecules bound to DOC are assumed to be not bioavailable and thus, do not contribute to $EC50_{total}$.

2.3.2 Model calibration and selection

The bioavailability model described above was calibrated with the toxicity data obtained from experiments, using function 'optim' in RStudio[30]. Because $fAMP^+$ and $fCLA^-$ are < 1% in the tested pH range, EC50(AMP^+) and EC50(CLA^-) were excluded from the calibration process. The calibration was performed for each test chemical, based on three different model assumptions: (1) both the neutral or zwitterionic and the charged species are toxic, (2) only the neutral or zwitterionic ($AMP^{+/-}$, CLA^0) species are toxic, and (3) only the charged species (AMP^-, CLA^+) are toxic.

Calibration employed the sum of squared errors (SSE) between the $EC50_{total}$ observed in experiments and that predicted by the bioavailability models to describe the 'goodness of fit' of

parameter values. The parameter values giving the lowest SSE were reported as 'optim'. The performance of the three models was evaluated using the Pearson's correlation analysis between $EC50_{total}$ predictions and observations. Details on the parameterization process and results can be found in Section S4 in supplementary data.

2.4 Statistical analysis

Data analyses were performed with RStudio Version 1.3.959[28] and Microsoft Excel. The analyses employed the mean of measured pH, the mean of measured DOC concentration, and the nominal concentrations of AMP and CLA as input. To obtain a dose-response relationship between the test chemical and the response of *M. aeruginosa*, a 3-parameter log-logistic dose-response relationship (Eq. 3) was fitted to the cell growth rate (r_{Gr}) using package *drc*[29] in RStudio. Negative growth rates (indicating cell death), when appeared, were removed from the analysis. The full 3-parameter dose-response curves of the test chemicals in Exp I-IV are shown in Figure S2-S5.

$$r_{Gr,treatment} = \frac{r_{Gr,max}}{1+\left(\frac{[C]}{96h\ EC50}\right)^{s}} \quad \text{Eq. 5}$$

where $r_{Gr,treatment}$ is the cell growth rate of *M. aeruginosa* (day^{-1}) in a treatment group, $r_{Gr,max}$ is the cell growth rate of *M. aeruginosa* (day^{-1}) in the control, [C] is the concentration of test chemical in the exposure vessel, 96h EC50 is the 50% effect concentration obtained from a 96-hour toxicity test, and s is the slope factor (-) in the dose-response relationship.

3. RESULTS

3.1 Water chemistry

In the four toxicity experiments, pH was maintained within $\pm$ 0.4 the nominal pH levels (Table 1; Table S1), which are the target pH levels of the test media. In Exp II and IV, DOC was within the nominal DOC level $\pm$ 2.0 mg L^{-1}. In Exp V, the mean pH was 7.4 $\pm$ 0.2 in media A and B, and 9.0 $\pm$ 0.2 in media C and D. The mean DOC concentration was 18.1 $\pm$ 0.2 $mg.L^{-1}$ in media B and D.

The measured CLA concentration in the control groups was below the LOQ (0.17 μg L^{-1}) (Table S4). In the rest groups, the average deviation between the nominal and the measured CLA concentration was 25%. Out of the 20 samples selected for analysis, 16 samples had a deviation below 30%. The average loss of CLA during the toxicity tests was 19%. Overall, CLA concentrations in the exposure vessels were in good agreement with the nominal concentrations. Hence, data analysis employed the nominal concentrations of CLA.

The measured AMP concentration in the control groups was below the quantification limit (LOQ = 4.42 μg L^{-1}) (Table S4). The average deviation between the measured and the nominal AMP concentration was 17%, and 25 of 29 samples showed a deviation < 30%. The average loss of AMP during the 96-hour toxicity tests was 38%. Thus, Exp V was conducted to give more information about AMP degradation in the absence of *M. aeruginosa*. The degradation of AMP in Exp V showed no pH-dependency, albeit it appeared to be faster in the presence of DOC. When DOC was absent, the loss of AMP during the 96-hour experiment was below 10% (Table S5), but in the presence of 20 mg L^{-1} DOC, the loss of AMP exceeded 40%, suggesting an average degradation rate constant of 0.15 day^{-1} when quantified as a first order degradation process. With the above information, we decided to use the nominal concentration of AMP for representing the AMP concentration to which the test organisms were exposed to at the start of the experiment. The potential impact introduced by DOC on the degradation of AMP is discussed in the Discussion.

3.2 Ecotoxicity of the test chemicals and correlation analysis

The 96h $EC50_{total}$ of AMP showed a clear pH-dependency. In Exp I, it increased from 10.7 μg L^{-1} at pH 7.5 to 51.3 μg L^{-1} at pH 9.0 (Figure 1, Table 1), correlating positively (R = 1.0, p-value < 0.05, Pearson's correlation) with pH (Table S2). A similar pattern can be observed in Exp II, in which the 96h $EC50_{total}$ of AMP at pH 9.0 (58.8 μg L^{-1}) quadrupled compared to that at pH 7.5 (16.4 μg L^{-1}), when no SRNOM was present. In general, AMP toxicity decreased with increasing pH, as $AMP^{+/-}$ transforms to AMP^{-}, which agreed with our first hypothesis.

In Exp III, the $EC50_{total}$ of CLA was between 3.7 μg L^{-1} and 5.1 μg L^{-1} in the pH range 7.3-9.0. The $EC50_{total}$ of CLA was the lowest at pH 8.5. No significant linear correlation was identified between CLA toxicity and pH using Pearson's correlation (Table S2), which conflicted our first hypothesis. The $EC50_{total}$ of CLA in Exp IV in media without SRNOM was close to those found in Exp III, but it was higher at pH 7.4 (8.3 μg L^{-1}) than at pH 8.3 (3.4 μg L^{-1}) or pH 9.0 (4.9 μg L^{-1}).

Comparing the $EC50_{total}$ observations in the test media with different levels of DOC, in Exp II and IV, neither the toxicity of AMP nor that of CLA was influenced by changes in DOC concentration (Figure 1). Correlation analysis identified no significant relationships between the $EC50_{total}$ of AMP and DOC concentration, or between the $EC50_{total}$ of CLA and DOC concentration (Table S2). In summary, DOC showed insignificant impact on AMP toxicity and CLA toxicity, which rejected our second hypothesis.

3.3 Bioavailability model simulation

Because DOC showed no influence on the $EC50_{total}$ of the test chemicals, equations in the bioavailability model relating to API-DOC binding were excluded from calibration. Data collected from Exp I and III were employed to parameterize the bioavailability model.

When assuming that both the charged and the neutral/zwitterionic species are toxic, the zwitterionic $AMP^{+/-}$ (EC50 = 3.97 μg L^{-1}) was predicted to be 15 times more toxic than the negatively charged AMP^{-} (EC50 = 59.9 μg L^{-1}) (Table S6), whereas the toxicity of CLA^{0} (EC50 = 4.22 μg L^{-1}) and CLA^{+} (EC50 = 6.12 μg L^{-1}) were predicted to be almost the same (Table S8). To simulate the situation in which all toxic effects are attributed to charged species, the EC50 of $AMP^{+/-}$ and CLA^{0} were set as 10^{15} μg L^{-1}. In this case, the corresponding EC50 of AMP^{-} and CLA^{+} were 23.4 μg L^{-1} and 3.69 μg L^{-1}, respectively. When the molecules carrying zero net charge are assumed as the only contributor to the overall toxicity, the estimated EC50 of $AMP^{+/-}$ and CLA^{0} were predicted at 0.74 μg $L^{-1}$and 0.30 μg L^{-1}, respectively.

The $EC50_{total}$ predictions made based on the different assumptions were compared with experimental observations (Figure 2). For AMP, the assumption that both the zwitterionic $AMP^{+/-}$

297 and negatively charged AMP^- are toxic can best simulate the actual changes in $EC50_{total}$
298 observations. Predictions based on this assumption were in good agreement ($R = 1.0$, $p < 0.05$,
299 Pearson's correlation) with the observed values (Table S6). For CLA, none of the three
300 assumptions can completely reproduce the trend in the observed $EC50_{total}$ ($p > 0.05$). Nonetheless,
301 predictions closest to the observations were made on the basis that both CLA^+ and CLA^0 are toxic,
302 and the estimated individual toxicity of the two species were similar (Table S8).

303 **4. DISCUSSION**

304 In this study, we used AMP and CLA as model molecules to investigate the influence of pH
305 and DOC on the bioavailability and toxicity of penicillin and macrolide antibiotics in freshwater.
306 pH has a strong impact on AMP toxicity that can be explained by speciation, whereas for CLA,
307 the influence of pH is relatively small and cannot be related to its speciation. The toxicity of AMP
308 or CLA is not affected by the presence of DOC.

309 4.1 Regulating bioavailability via pH-driving chemical speciation

310 The impact of pH on AMP bioavailability shows clear linkage with speciation. In order for
311 AMP to function, it must first penetrate the outer layer of bacteria, the cell wall and the membrane
312 system[31]. The test organism *M. aeruginosa* is a gram-negative bacterium. The cell membrane
313 allows *M. aeruginosa* to maintain a stable intercellular pH regardless of variations in extracellular
314 pH to a certain extent[32]. Meanwhile, the porin channels on the membrane allow certain molecules
315 to go through the membrane barrier by passive diffusion via nonspecific porins[33,34]. Yet, whether
316 or not a molecule can make use of the porin channels is affected by its molecular size and
317 polarity[33,34]. For instance, a molecule with PSA > 140 $Å^2$ is considered too polar to permeate the
318 membrane of human cells[35]. Although the exact polarity boundary of the membrane of *M.*
319 *aeruginosa* is unknown, $AMP^{+/-}$ is favored by the porin channels in some gram-negative bacteria
320 and has a higher translocation rate than compounds with one or more negative charge(s), e.g.,
321 piperacillin[34,36]. Hence, when $AMP^{+/-}$ is the dominant species, the molecules are most bioavailable,
322 and the highest ecotoxicity of AMP is observed.

Although CLA also shifts between the positively charged CLA^+ and the neutral CLA^0 in our tested pH range, changes in the total toxicity of CLA cannot be attributed to changes in speciation. The outer membrane of gram-negative bacteria is an effective barrier against CLA[24]. In addition, APIs known to be regulated by pH, e.g., ciprofloxacin[11], fluoxetine[37], sulfadiazine[9], and triclosan[38,39], and AMP in this study, often have a PSA < 140 Å². Compared to the APIs above, CLA has a higher PSA (183 Å²) and a large molecular size, which might restrict CLA from utilizing passive diffusion[40]. Thus, it is possible that CLA remains in the extracellular phase and affects *M. aeruginosa* by baseline toxicity instead of protein synthesis inhibition, or CLA might be taken by *M. aeruginosa* via facilitated or active transport rather than passive diffusion. In either case, the uptake of CLA by *M. aeruginosa*, and therefore the bioavailability of CLA, have limited dependency on the ionizing state. Generalizing the effect of pH-driving speciation on CLA bioavailability will result in an underestimation of the risk potentially caused by the chemical in aquatic environments.

4.2 Absent DOC effects on ecotoxicity

It is surprising to observe no influence of DOC on the ecotoxicity of either of the test chemicals, especially CLA. DOC has previously been associated with toxicity reduction of several compounds (Table 2), including erythromycin (ERY), a member of the macrolide family that shares a similar chemical structure and physical-chemical properties with CLA. Liu et al[41] found four types of DOC compounds, including Suwannee River fulvic acid (SRFA) and humic acid (SRHA), to pose inhibitory effects on the uptake of ERY by duckweed *Lemna minor* and adult zebrafish *Danio rerio*. The equilibrium concentration of ERY in the two organisms was lowered by respectively 11-26% in zebrafish and 28-41% in duckweed with the introduction of 20 mg L^{-1} DOC into the culture media.

In addition to ERY, DOC also affects the toxicity or bioavailability of chlorophenols to *Chlorella vulgaris*[42,43], ciprofloxacin to *Microcystis aeruginosa*[11,44], sertraline to *Danio rerio*[37], triclosan to *Danio rerio*[38] and *Gammarus pulex*[45], and tetracycline to *Escherichia coli*[46] (Table S3; Figure S7). These chemicals vary considerably in their chemical and physical properties. For

instance, their polar surface area (PSA, ranges from 12 to 194 Å^2) and hydrophobicity (logP ranges from -1.73 to 5.51) cover a wide range of values (Table 2). This suggests that both electrostatic interactions and hydrophobic interactions may play a role in the interactions between DOC and the aforementioned chemicals. Indeed, at environmentally relevant pH (e.g., pH 6.0-10.0), Suzuki and Shoji[42] suggest that both types of interactions are relevant for the interactions between DOC and chlorophenols (logP = 3.06, 3.69), whereas Aristilde and Sposito[47] show that various electrostatic forces such as hydrogen bonding, π-π stacking, ionic interactions, all contribute to the binding between ciprofloxacin (logP = -0.28) and DOC. Hence, no single property seems to act as a decisive predictor of the impact of DOC on bioavailability, but the whole set of physical-chemical properties of the ionizable chemicals such as hydrophobicity, ionizing states, and molecular structure, need to be taken into consideration when evaluating the role of DOC.

For ERY, the primary force that binds the chemical and DOC is the interaction between ERY^+ and deprotonated carboxylate groups COO^- on DOC, which is facilitated by hydrophobic partitioning and hydrogen bonding[41]. CLA, along with other structurally similar macrolide antibiotics, have been suggested to share common binding sites on aquatic DOC as ERY[41,48]. CLA merely differs from ERY by the replacement of the 6-hydroxy group with a 6-methoxy group. It is, however, unclear, whether or not this difference is sufficient to reduce interaction strength of CLA with DOC sufficiently such that DOC does not affect CLA toxicity, as observed in the present study.

The strength of DOC-CLA interactions is also influenced by pH and ionic strength, too. According to Sibley and Pedersen (2008)[48], the binding between CLA and Elliot soil humic acid (ESHA) decreases with increasing ionic strength, whereas at a constant ionic strength, the CLA-ESHA binding decreases with increasing pH when pH exceeds 6.5[48]. Therefore, it cannot be ruled out that differences in pH and ionic strength play a role in the different effects of DOC on ERY toxicity versus CLA toxicity.

It is also noteworthy that the type of DOC can significantly affect DOC-API interactions. For instance, while the three different DOC compounds (coal-derived, autochthonous, terrigenous) investigated by Alsop and Wilson[37] showed no influence on the ecotoxicity of ethinyl estradiol, Wu et al[49] found Aldrich humic acid to interact strongly with ethinyl estradiol. Zhang et al[44] also demonstrated that the binding potency of ciprofloxacin to humic acid (2022 L g^{-1}) is nearly twice that of fulvic acid (1041 L g^{-1}) extracted from the same source (Schwarzbach stream, Küchelscheid, Belgium). Nonetheless, we consider it not likely that the specific composition of the DOC used in the present study can account for the absence of DOC effect observed in our tests, because the carboxyl group charge densities of SRHA (9.59 meg g^{-1} C) and SRFA (11.17 meg g^{-1} C) in Liu et al (2019)[41], and SRNOM (11.21 meg g^{-1} C) in our study, are comparable (http://humic-substances.org/acidic-functional- groups-of-ihss-samples/).

Furthermore, the different routes of uptake of *Lemna minor*, *Danio rerio*, and *Microcystis aeruginosa*, through the organism-water interface may also contribute to the absence of DOC effect. For instance, if the uptake pathway of CLA by *M. aeruginosa* is facilitated or active transport as discussed in the previous section, the concentration of free CLA may not be the determining factor that regulates CLA uptake. In this case, the strength of the CLA-DOC interactions could have limited impact on CLA ecotoxicity, as we observed in the present study.

As for the AMP, there remains a lack of understanding of the interactions between DOC and β-lactams[50]. In previous studies conducted by Aristilde and Sposito (2010, 2013)[51,52], it is shown that zwitterion ciprofloxacin (log P = -0.28) interacts with DOC via electrostatic interactions, such as van der Waals interactions. Considering the low hydrophobicity of AMP (log P = 1.35), electrostatic interactions may have a greater impact than hydrophobicity interactions on AMP-DOC interactions as well. In the pH range examined in our study, the dominant forms of AMP are $AMP^{+/-}$ and AMP^{-}. It is possible that these zwitterions and negatively charged anions interact only weakly with the deprotonated functional groups on DOC, which limits the effect of DOC on AMP ecotoxicity.

4.3 Slower cell growth, milder toxic effect?

Rather than pH or DOC, the $EC50_{total}$ observations of CLA across Exp III and IV demonstrate a significant correlation with *M. aeruginosa* cell growth rate in the control ($R = -0.88$, p-value < 0.05). Imperfect pH control and natural variations in *M. aeruginosa* occasionally resulted in comparatively slower cell growth in some test media, which allows us to identify the parallel changes in the environmental pH and the toxicity of CLA (Figure S6). In the test media with relatively low cell growth rate (0.5-0.6 day^{-1}), the $EC50_{total}$ of CLA was two times higher than in test media with control cell growth rates between 0.7 and 0.8 day^{-1}. The growth rate of *M. aeruginosa* might have an impact on CLA ecotoxicity, possibly by affecting cell membrane components and/or by regulating CLA uptake directly.

4.4 Implications for toxicity testing of ionizable compounds

Considering that pH is constantly changing in the toxicity test system, we imported two series of fluctuating pH (mean pH = 7.3 and 9.3) into the bioavailability model to simulate AMP EC50 in Exp I. At the pH 7.3 scenario, an additional scenario where 20 mg L^{-1} DOC is present was added (pH 7.3 + DOC), in which AMP degrades at a rate constant of 0.15 day^{-1}. In the other two scenarios, AMP degradation is not considered because the loss due to degradation is less than 10% in the absence of DOC. Figure 3A shows the pH input employed by model simulation. The input was based on the pH measured during Exp I and the assumption that pH changed linearly between the two measurements. Using the EC50 of AMP estimated for pH 7.3 (8.9 μg L^{-1}) and 9.3 (55.2 μg L^{-1}) as input, respectively, the time course of the relative effect (RE) of AMP on *M. aeruginosa* is shown in Figure 3B.

The most drastic fluctuation in the pH input at both the lower range (pH 7.3) and the higher range (pH 9.3) reaches 0.7, but the actual course of impacts on *M. aeruginosa* cell growth rate caused by the pH variations differs by more than 2-fold. The 10.9 μg L^{-1} increase in AMP EC50 (pH 7.2-7.9) leads to a decrease of RE from 58.6% to 8.6%, whereas the 12.5 μg L^{-1} decrease of AMP EC50 (pH 9.3-8.6) results in only 20.1% change in the relative response. Hence, the impact of pH on AMP ecotoxicity is more pronounced at a pH close to its pKa_2 (7.14), than at a pH more distant from the pKa value, i.e., pH above 8.5. In the risk assessment of ionizable organic

chemicals, when the pKa of a chemical is close to the environmental pH, it becomes more important to consider the impact of pH.

4.5 Implications from the DOC effect on AMP degradation

DOC often has a negative influence on the photodegradation of chemicals, via weakening light penetration and scavenging radicals, e.g., ·OH, both of which tend to slow down photodegradation[53]. However, phototransients derived from the photolysis of DOC compounds, e.g., DOC in excited triplet states, might also photosensitize the degradation of some organic chemicals[54]. An example is bisphenol A, the photodegradation of which is enhanced by the presence of Suwannee River fulvic acid[55]. Metal ions entering test media together with DOC might play a role, too. Cobalt (Co(II)), zinc (Zn(II)) and cadmium (Cd(II)) catalyze the hydrolysis of β-1actams[56], and iron species (e.g., Fe(II) and Fe(III) decompose hydrogen peroxide (e.g., H_2O_2) via Fenton reactions to promote the photodegradation of β-1actams[57,58].

Theoretically, a reduction in AMP concentration reduces the toxic effects caused by the chemical. For instance, in our simulation (Figure 3B), AMP concentration decreases by 36% in 3-day in the presence of DOC. As a result, the actual effect caused by AMP at pH 7.3 after 3 days is only 23%, less than half of that when no degradation occurs under the same conditions (59%). However, in our experiments, AMP ecotoxicity in test media at the same pH level remains steady despite differences in DOC concentration. A possibility is that AMP uptake by *M. aeruginosa* was fast and toxic effects were triggered before AMP degradation could have had a significant influence. In order to quantify the impact of degradation, the uptake and elimination rate of AMP by organisms are factors to consider in future toxicity experiments involving DOC.

5. CONCLUSIONS

Our findings show that pH is an important factor to consider in the risk assessment of certain ionizable chemicals, including AMP. At pH close to the acid dissociation constants of APIs, where drastic changes in speciation are expected, the influence of pH is especially pronounced. However, the impact of pH-driven speciation on bioavailability cannot be extrapolated and applied to ionizable antibiotics in general, as CLA ecotoxicity remains stable across the pH range

7.3-9.3. Neither can the influence of DOC be generalized. Both AMP and CLA toxicity appear to experience a significant impact from DOC in our tested pH range, whereas earlier work on ciprofloxacin clearly indicated an inhibitory effect of DOC on ciprofloxacin ecotoxicity across the pH range 7.2-9.0. Apart from speciation, other physical-chemical properties of the antibiotics – such as molecular size and polarity – may have a significant impact on the bioavailability of the chemicals, albeit the linkage awaits further exploration. For small, relatively non-polar molecules such as AMP, pH may be more likely to play a role in regulating bioavailability, but for chemicals with a large molecule size like CLA, bioavailability might be less relevant.

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REFERENCE

1. Wilkinson, J. L., Boxall, A. B., Kolpin, D. W., Leung, K. M., Lai, R. W., Galbán-Malagón, C., ... & Teta, C. (2022). Pharmaceutical pollution of the world's rivers. *Proceedings of the National Academy of Sciences*, *119*(8), e2113947119.
2. Zhang, C., Yu, Z. G., Zeng, G. M., Jiang, M., Yang, Z. Z., Cui, F., ... & Hu, L. (2014). Effects of sediment geochemical properties on heavy metal bioavailability. *Environment international*, *73*, 270-281.
3. Bittner, L., Teixidó, E., Keddi, I., Escher, B. I., & Klüver, N. (2019). pH-dependent uptake

and sublethal effects of antihistamines in zebrafish (*Danio rerio*) embryos. *Environmental toxicology and chemistry*, *38*(5), 1012-1022.

4. Rizzuto, S., Thrane, J. E., Baho, D. L., Jones, K. C., Zhang, H., Hessen, D. O., ... & Leu, E. (2020). Water browning controls adaptation and associated trade-offs in phytoplankton stressed by chemical pollution. *Environmental Science & Technology*, *54*(9), 5569-5579.

5. Comer, J., & Tam, K. (2001). Lipophilicity profiles: theory and measurement. *Pharmacokinetic optimization in drug research: biological, physicochemical, and computational strategies*, 275-304.

6. Manallack, D. T. (2007). The p K a distribution of drugs: application to drug discovery. *Perspectives in medicinal chemistry*, *1*, 1177391X0700100003.

7. Steinberg, T. H. (1994). Cellular transport of drugs. *Clinical infectious diseases*, *19*(5), 916-921.

8. Shore, P. A., Brodie, B. B., & Hogben, C. A. M. (1957). The gastric secretion of drugs: a pH partition hypothesis. *Journal of Pharmacology and Experimental Therapeutics*, *119*(3), 361-369.

9. Anskjær, G. G., Rendal, C., & Kusk, K. O. (2013). Effect of pH on the toxicity and bioconcentration of sulfadiazine on *Daphnia magna*. *Chemosphere*, *91*(8), 1183-1188.

10. Neuwoehner, J., & Escher, B. I. (2011). The pH-dependent toxicity of basic pharmaceuticals in the green algae *Scenedesmus vacuolatus* can be explained with a toxicokinetic ion-trapping model. *Aquatic toxicology*, *101*(1), 266-275.

11. Zhang, Q., Demeestere, K., & De Schamphelaere, K. A. (2022). A Bioavailability Model to Predict the Impact of pH and Dissolved Organic Carbon on Ciprofloxacin Ecotoxicity to the Cyanobacteria *Microcystis aeruginosa*. *Environmental Toxicology and Chemistry*.

12. Hamelink, J., Landrum, P. F., Bergman, H., & Benson, W. H. (1994). Bioavailability: physical, chemical, and biological interactions. CRC press.

13. Chalew, T. E., & Halden, R. U. (2009). Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban 1. *JAWRA Journal of the American Water Resources Association*, *45*(1), 4-13.

14. De Paolis, F., & Kukkonen, J. (1997). Binding of organic pollutants to humic and fulvic acids: influence of pH and the structure of humic material. *Chemosphere*, *34*(8), 1693-1704.

15. McCarthy, J. F. (1989). Bioavailability and toxicity of metals and hydrophobic organic

contaminants. I.H. Suffet, P. MacCarthy (Eds.), Aquatic humic substances: influence on fate and treatment of pollutants, American Chemical Society, Washington DC (1989), pp.263-277.

16. Aristilde, L., & Sposito, G. (2013). Complexes of the antimicrobial ciprofloxacin with soil, peat, and aquatic humic substances. *Environmental toxicology and chemistry*, *32*(7), 1467-1478.

17. Behera, S. K., Oh, S. Y., & Park, H. S. (2010). Sorption of triclosan onto activated carbon, kaolinite and montmorillonite: effects of pH, ionic strength, and humic acid. *Journal of hazardous materials*, *179*(1-3), 684-691.

18. Vitale, C. M., & Di Guardo, A. (2019). A review of the predictive models estimating association of neutral and ionizable organic chemicals with dissolved organic carbon. *Science of the Total Environment*, *666*, 1022-1032.

19. Bronzwaer, S. L., Cars, O., Buchholz, U., Mölstad, S., Goettsch, W., Veldhuijzen, I. K., ... & Degener, J. E. (2002). The relationship between antimicrobial use and antimicrobial resistance in Europe. *Emerging infectious diseases*, *8*(3), 278.

20. Congilosi, J. L., & Aga, D. S. (2021). Review on the fate of antimicrobials, antimicrobial resistance genes, and other micropollutants in manure during enhanced anaerobic digestion and composting. *Journal of Hazardous Materials*, *405*, 123634.

21. Acred, P., Brown, D. M., Turner, D. H., & Wilson, M. J. (1962). Pharmacology and chemotherapy of ampicillin—a new broad-spectrum penicillin. *British Journal of Pharmacology and chemotherapy*, *18*(2), 356-369.

22. Nathwani, D., & Wood, M. J. (1993). Penicillins. *Drugs*, *45*(6), 866-894.

23. Peters, D. H., & Clissold, S. P. (1992). Clarithromycin. *Drugs*, *44*(1), 117-164.

24. Delcour, A. H. (2009). Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1794*(5), 808-816.

25. Guillard, R. R., & Lorenzen, C. J. (1972). Yellow-green algae with chlorophyllide C. *Journal of Phycology, 8*(1), 10-14.

26. Organisation for Economic Co-operation and Development (OECD) (2011), *Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264069923-en.

27. Salminen, R., Batista, M., Bidovec, M., Demetriades, A., De Vivo, B., De Vos, W., . . . Halamic, J. (2005). Part 1. Background Information, Methodology, and Maps. *Geochemical Atlas of Europe; De Vos, W., Tarvainen, T., Eds*.

28. R Foundation for Statistical Computing. (2019). R: A language and environment for statistical computing. https://www.R-project.org/.

29. Ritz, C., & Strebig, J. (2016). Package "drc." R Foundation for Statistical Computing.

30. Ghalanos, A., & Theussl, S. (2012). Package "Rsolnp." R Foundation for Statistical Computing.

31. Raynor, B. D. (1997). Penicillin and ampicillin. *Primary Care Update for OB/GYNS*, *4*(4), 147-152.

32. Guan, N., & Liu, L. (2020). Microbial response to acid stress: mechanisms and applications. *Applied microbiology and biotechnology*, *104*(1), 51-65.

33. Kowata, H., Tochigi, S., Takahashi, H., & Kojima, S. (2017). Outer membrane permeability of cyanobacterium *Synechocystis sp. strain PCC 6803*: studies of passive diffusion of small organic nutrients reveal the absence of classical porins and intrinsically low permeability. *Journal of bacteriology*, *199*(19), e00371-17.

34. Danelon, C., Nestorovich, E. M., Winterhalter, M., Ceccarelli, M., & Bezrukov, S. M. (2006). Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. *Biophysical journal*, *90*(5), 1617-1627.

35. Matsson, P., & Kihlberg, J. (2017). How big is too big for cell permeability?. *Journal of Medicinal Chemistry*, *60*(5), 1662-1664.

36. Yoshimura, F., & Nikaido, H. (1985). Diffusion of beta-lactam antibiotics through the porin channels of *Escherichia coli K-12*. *Antimicrobial agents and chemotherapy*, *27*(1), 84-92.

37. Alsop, D., & Wilson, J. Y. (2019). Waterborne pharmaceutical uptake and toxicity is modified by pH and dissolved organic carbon in zebrafish. *Aquatic Toxicology*, *210*, 11-18.

38. Rowett, C. J., Hutchinson, T. H., & Comber, S. D. (2016). The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on the toxicity of triclosan to the crustacean *Gammarus pulex* (L.). *Science of the Total Environment*, *565*, 222-231.

39. Li, C., Qu, R., Chen, J., Zhang, S., Allam, A. A., Ajarem, J., & Wang, Z. (2018). The pH-dependent toxicity of triclosan to five aquatic organisms (*Daphnia magna*, *Photobacterium phosphoreum*, *Danio rerio*, *Limnodrilus hoffmeisteri*, and *Carassius*

auratus). *Environmental Science and Pollution Research*, *25*(10), 9636-9646.

40. Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and molecular biology reviews*, *67*(4), 593-656.

41. Liu, Z., Delgado-Moreno, L., Lu, Z., Zhang, S., He, Y., Gu, X., ... & Wang, W. (2019). Inhibitory effects of dissolved organic matter on erythromycin bioavailability and possible mechanisms. *Journal of hazardous materials*, *375*, 255-263.

42. Suzuki, D., & Shoji, R. (2020). Toxicological effects of chlorophenols to green algae observed at various pH and concentration of humic acid. *Journal of Hazardous Materials*, *400*, 123079.

43. Xing, L., Liu, H., Giesy, J. P., & Yu, H. (2012). pH-dependent aquatic criteria for 2, 4-dichlorophenol, 2, 4, 6-trichlorophenol and pentachlorophenol. S*cience of the total environment*, 441, 125-131.

44. Zhang, Q., Demeestere, K., & De Schamphelaere, K. A. (2023). Water brownness regulates the bioavailability of a fluoroquinolone antibiotic: UV-absorbance as a predictor of ciprofloxacin ecotoxicity. *Environmental pollution*, *334*,122209.

45. Carmosini, N., Grandstrand, S., & King-Heiden, T. C. (2016). Developmental toxicity of triclosan in the presence of dissolved organic carbon: moving beyond standard acute toxicity assays to understand ecotoxicological risk. *Zebrafish*, *13*(5), 424-431.

46. Chen, Z., Zhang, Y., Gao, Y., Boyd, S. A., Zhu, D., & Li, H. (2015). Influence of dissolved organic matter on tetracycline bioavailability to an antibiotic-resistant bacterium. *Environmental science & technology*, *49*(18), 10903-10910.

47. Aristilde, L., & Sposito, G. (2013). Complexes of the antimicrobial ciprofloxacin with soil, peat, and aquatic humic substances. *Environmental toxicology and chemistry*, *32*(7), 1467-1478.

48. Sibley, S. D., & Pedersen, J. A. (2008). Interaction of the macrolide antimicrobial clarithromycin with dissolved humic acid. *Environmental science & technology*, *42*(2), 422-428.

49. Wu, C., Zhang, K., Huang, X., & Liu, J. (2016). Sorption of pharmaceuticals and personal care products to polyethylene debris. *Environmental Science and pollution research*, *23*, 8819-8826.

50. Klein, A. R., Sarri, E., Kelch, S. E., Basinski, J. J., Vaidya, S., & Aristilde, L. (2021). Probing

608 the fate of different structures of beta-lactam antibiotics: hydrolysis, mineral capture, and
609 influence of organic matter. *ACS Earth and Space Chemistry*, *5*(6), 1511-1524.

610 51. Aristilde, L., & Sposito, G. (2010). Binding of ciprofloxacin by humic substances: a
611 molecular dynamics study. *Environmental Toxicology and Chemistry*, *29*(1), 90-98.

612 52. Aristilde, L., & Sposito, G. (2013). Complexes of the antimicrobial ciprofloxacin with soil,
613 peat, and aquatic humic substances. *Environmental toxicology and chemistry*, *32*(7), 1467-
614 1478.

615 53. Landrum, P. F., Nihart, S. R., Eadie, B. J., & Herche, L. R. (1987). Reduction in
616 bioavailability of organic contaminants to the amphipod *Pontoporeia hoyi* by dissolved
617 organic matter of sediment interstitial waters. *Environmental Toxicology and Chemistry: An*
618 *International Journal*, *6*(1), 11-20.

619 54. Wenk, J., Von Gunten, U., & Canonica, S. (2011). Effect of dissolved organic matter on the
620 transformation of contaminants induced by excited triplet states and the hydroxyl
621 radical. *Environmental science & technology*, *45*(4), 1334-1340.

622 55. Chin, Y. P., Miller, P. L., Zeng, L., Cawley, K., & Weavers, L. K. (2004). Photosensitized
623 degradation of bisphenol A by dissolved organic matter. *Environmental science &*
624 *technology*, *38*(22), 5888-5894.

625 56. Beard, S. J., Ciccognani, D. T., Hughes, M. N., & Poole, R. K. (1992). Metal ion-catalysed
626 hydrolysis of ampicillin in microbiological growth media. *FEMS microbiology letters*, *96*(2-
627 3), 207-211.

628 57. Sun, C., Chen, C., Ma, W., & Zhao, J. (2011). Photodegradation of organic pollutants
629 catalyzed by iron species under visible light irradiation. *Physical Chemistry Chemical*
630 *Physics*, *13*(6), 1957-1969.

631 58. Kumar, A., Rana, A., Sharma, G., Naushad, M., Dhiman, P., Kumari, A., & Stadler, F. J.
632 (2019). Recent advances in nano-Fenton catalytic degradation of emerging pharmaceutical
633 contaminants. *Journal of Molecular Liquids*, *290*, 111177.

634

635 **Table 1**. Measured water chemistry factors (pH (n=5) and DOC (n=4)) and toxicity data: median
636 effect concentration (EC50) with 95% confidence interval, slope of the 3-parameter dose-
637 response curve, and maximum cell growth rate (Gr_{max}) of ampicillin (AMP) and clarithromycin
638 (CLA).

Experiment	Chemical	Mean pH ± SD[a] (-)	DOC ± SD (mg L^{-1})	96h EC50 (µg L^{-1})	Slope ± SD (-)	Gr_{max} ± SD (day^{-1})
I	AMP	7.5 ± 0.3	0.3 ± 0.1	10.7 (8.3, 11.8)	9.8± 7.7	0.75 ± 0.01
		8.0 ± 0.4	0.2 ± 0.1	24.7 (23.2, 26.3)	3.6 ± 0.3	0.75 ± 0.01
		8.4 ± 0.2	0.4 ± 0.2	33.6 (30.4, 36.8)	3.9 ± 0.7	0.72 ± 0.01
		9.0 ± 0.3	0.5 ± 0.6	51.3 (47.9, 54.7)	3.4 ± 0.3	0.72 ± 0.01
II	AMP	7.5 ± 0.3	0.3 ± 0.2	16.4 (15.4, 17.3)	3.8 ± 0.4	0.76 ± 0.01
		7.4 ± 0.3	9.4 ± 0.4	14.5 (13.3, 15.7)	2.7 ± 0.3	0.81 ± 0.01
		7.4 ± 0.3	19.0 ± 0.5	14.7 (13.3, 16.0)	3.5 ± 0.4	0.81 ± 0.01
		9.0 ± 0.4	0.3 ± 0.1	58.8 (55.3, 62.3)	2.9 ± 0.2	0.79 ± 0.01
		9.1 ± 0.3	9.3 ± 0.1	52.9 (50.1, 55.8)	2.8 ± 0.2	0.82 ± 0.01
		9.1 ± 0.3	18.3 ± 0.4	55.2 (51.0, 59.5)	2.9 ± 0.3	0.82 ± 0.01
III	CLA	7.3 ± 0.2	0.3 ± 0.4	4.6 (4.2, 5.1)	2.1 ± 0.2	0.77 ± 0.01
		7.5 ± 0.2	0.3 ± 0.3	4.3 (3.9, 4.6)	2.0 ± 0.2	0.75 ± 0.01
		7.9 ± 0.1	0.0 ± 0.1	4.0 (3.7, 4.3)	2.1 ± 0.1	0.75 ± 0.01
		8.5 ± 0.1	0.1 ± 0.0	3.7 (3.5, 4.0)	2.2 ± 0.1	0.73 ± 0.01
		9.0 ± 0.3	0.3 ± 0.2	5.1 (4.8, 5.3)	2.4 ± 0.1	0.73 ± 0.01
IV	CLA	7.4 ± 0.1	0.2 ± 0.4	8.3 (7.5, 9.2)	2.0 ± 0.2	0.56 ± 0.01
		7.4 ± 0.1	9.2 ± 0.2	8.7 (8.3, 9.1)	2.8 ± 0.1	0.59 ± 0.00
		7.4 ± 0.1	19.2 ± 0.2	7.7 (7.0, 8.3)	2.5 ± 0.2	0.58 ± 0.01
		8.3 ± 0.2	0.2 ± 0.2	3.4 (3.1, 3.6)	2.8 ± 0.2	0.71 ± 0.01
		8.3 ± 0.2	9.4 ± 0.5	3.9 (3.6, 4.2)	2.3 ± 0.2	0.74 ± 0.01
		8.3 ± 0.2	19.2 ± 0.2	4.0 (3.7, 4.3)	1.8 ± 0.1	0.81 ± 0.01
		9.0 ± 0.3	0.2 ± 0.2	3.9 (3.7, 4.1)	2.5 ± 0.1	0.72 ± 0.01
		9.0 ± 0.3	9.0 ± 0.5	3.9 (3.7, 4.0)	2.1 ± 0.1	0.78 ± 0.01
		9.0 ± 0.3	19.1 ± 0.2	4.0 (3.9, 4.2)	2.2 ± 0.1	0.82 ± 0.01

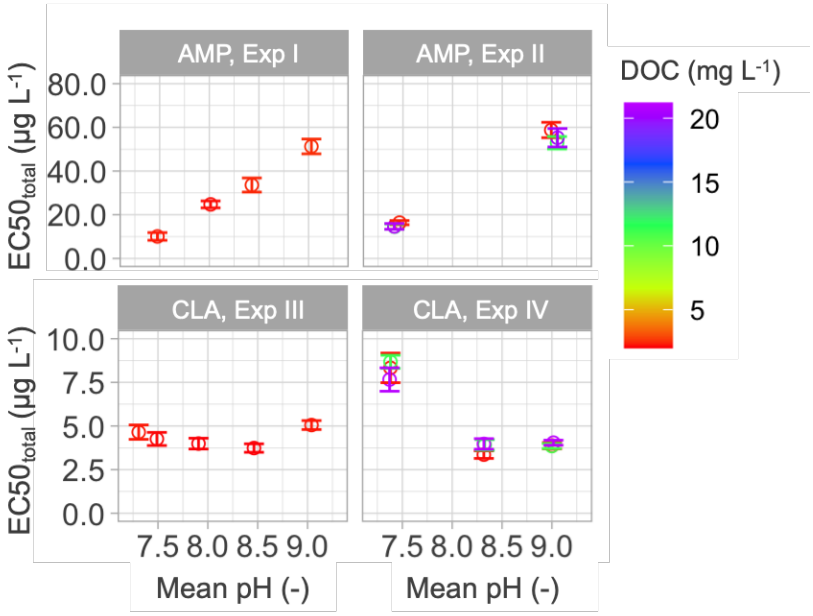
639 [a] SD= standard deviation

640 **Table 2**. The physical-chemical properties and toxicity information of the chemicals investigates by this study and ionizable chemicals that are known to be influenced

641 by pH and dissolved organic carbon (DOC). More detailed toxicity data is listed in Table S3 in the supplemental file.

Chemical	Molar mass ($g\ mol^{-1}$)	pKa (-)	Log P (-)	Polar surface area ($Å^2$)	Organism	Effect	Impact of pH and DOC	Reference
Ampicillin	349.1	2.55, 7.14	1.35	138	*Microcystis aeruginosa*	Growth inhibition	pH-dependency	This study
Clarithromycin	748.0	8.99	3.16	183	*Microcystis aeruginosa*	Growth inhibition	pH-dependency	This study
Sulfadiazine	250.3	6.36	-0.09	106	*Daphnia magna*	Immobilization	pH-dependency	[9]
Fluoxetine	309.3	9.80	4.05	21	*Danio rerio*	Survival	pH-dependency	[37]
Erythromycin	733.9	8.88	3.06	194	*Lemna minor*	Uptake	Inhibitory effect of DOC	[41]
					Danio rerio	Uptake	Inhibitory effect of DOC	
2,4-dichlorophenol	163.0	7.89	3.06	20	*Chlorella vulgaris*	Growth inhibition	pH-dependency Effect of DOC	[42]
					Danio rerio	Immobilization	pH-dependency	[43]
2,4,6-trichlorophenol	197.4	6.23	3.69	20	*Chlorella vulgaris*	Growth inhibition	pH-dependency Inhibitory effect of DOC	[42]
					Danio rerio	Immobilization	pH-dependency	[43]
pentachlorophenol	266.3	4.70	5.12	20	*Danio rerio*	Immobilization	pH-dependency	[43]
Ciprofloxacin	331.4	6.95, 8.95	0.28	73	*Microcystis aeruginosa*	Growth inhibition	pH-dependency Inhibitory effect of DOC	[11, 44]
Sertraline	306.2	9.16	5.51	12	*Danio rerio*	Survival	pH-dependency Inhibitory effect of DOC	[37]
Triclosan	289.6	7.90	4.76	30	*Danio rerio*	Survival	pH-dependency	[45]
					Gammarus pulex	Immobilisation	pH-dependency Inhibitory effect of DOC	[38]
Tetracycline	444.4	3.3	-1.73	182	*Escherichia coli*	Antibiotic resistance response	Inhibitory effect of DOC	[46]

642 [a]The physical-chemical properties of the compounds were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov) and United State Environmental Protection Agency (https://www.epa.gov).

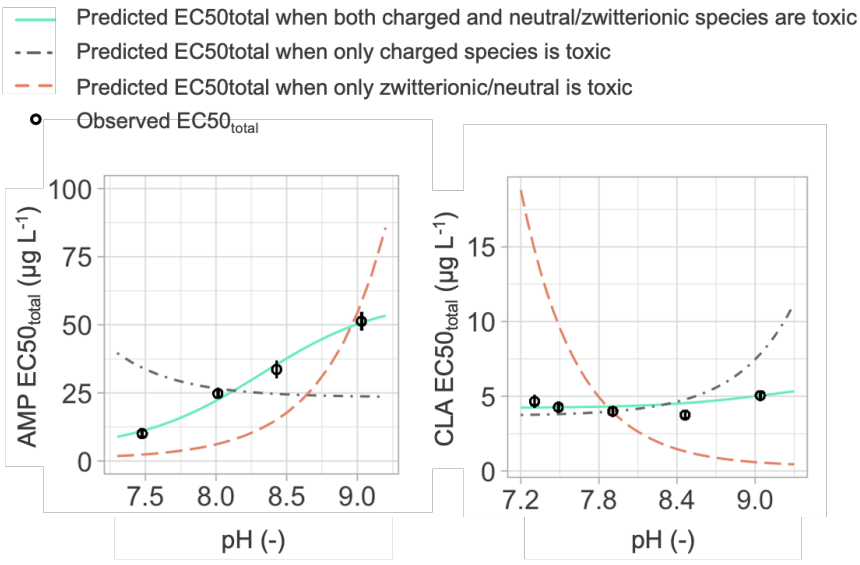


643

644 **Figure 1**. The 96-hour median effect concentration ($EC50_{total}$) of ampicillin and clarithromycin

645 plotted against the mean pH. Colour indicates the mean the concentration of dissolved organic

646 carbon (DOC) measured in the test medium.



647

648 **Figure 2**. The predicted $EC50_{total}$ of ampicillin (left) and clarithromycin (right) plotted against

649 experimental observations. Predictions were based on three assumptions: (1) both charged and

650 neutral/zwitterionic species are toxic, (2) only charged species are toxic, and (3) only the neutral

651 species are toxic. Empty circles represent observed $EC50_{total}$ with 95% confidence interval.

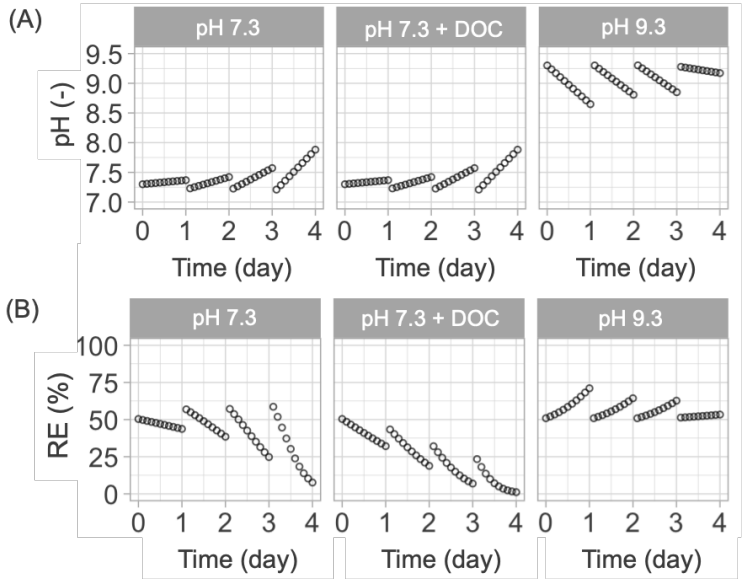


Figure 3. Model simulation of AMP ecotoxicity in test media with no DOC in Exp I. (A) The pH input used in model simulation. (B) The predicted relative effect (RE) of *Microcystis aeruginosa* to the EC50 of AMP estimated based on pH input (Table 1). The subtitles indicate the nominal pH level employed in the experiment. Input parameters: slope factor of dose-response relationship = 3.3. The degradation rate constant of AMP is 0.15 day^{-1} in the presence of DOC and 0 day^{-1} in the absence of DOC.