Abstract

Ciprofloxacin (CIP) is a pseudo-persistent antibiotic detected in freshwater worldwide. As an ionizable chemical, its fate in freshwater is influenced by water chemistry factors such as pH, hardness and dissolved organic carbon (DOC) content. Here, we investigated the effect of pH, DOC and Ca$^{2+}$ levels on the toxicity of CIP to *Microcystis aeruginosa*, and developed a bioavailability model on the basis of these experimental results. We found that the zwitterion (CIP$^{\pm}$) is the most bioavailable species of CIP to *M. aeruginosa*, whereas DOC is the most dominant factor reducing CIP toxicity, possibly via binding of both CIP$^{\pm}$ and CIP$^+$ to DOC. pH likely also regulates the CIP-DOC binding indirectly through its influence on CIP speciation. In addition, higher tolerance to CIP by *M. aeruginosa* was observed at pH < 7.2, but the underlying mechanism is yet unclear. Calcium was identified as an insignificant factor in CIP bioavailability. When parameterized with the data obtained from toxicity experiments, our bioavailability model is able to provide accurate predictions of CIP toxicity, as the observed and predicted total median effective concentration (EC50$_{\text{total}}$) deviated by less than 28% from each other. Our model predicts that changes in pH and DOC conditions can affect CIP toxicity by up to tenfold, suggesting that CIP in many natural environments is likely less toxic than in standard laboratory toxicity experiments.
1 Introduction

Ciprofloxacin (CIP) is a critically important antimicrobial (World Health Organization, 2016). Its appearance in aquatic environments originates from both human and veterinary medicine usage (Knapp et al., 2005; Van Doorslaer et al., 2014). As a pseudo-persistent antibiotic belonging to the fluoroquinolone (FQ) family, CIP is insensitive to biodegradation while prone to photo-induced degradation. The latter consists of both direct photolysis and indirect photolysis, which is due to reaction between CIP and reactive oxygen species (ROS) such as hydroxyl radicals (Al-Ahmad et al., 1999; Andreozzi et al., 2003; Ge et al., 2010). However, constituents of freshwater including humic acids (HA), Fe$^{3+}$ cations, NO$_3^-$ and Cl$^-$ anions can reduce the abundance of ROS in aqueous environments inhibiting the photo-induced degradation of FQs (Ge et al., 2010). These features allow CIP to remain in surface water and become a potential threat to aquatic organisms.

Risk assessment of pharmaceuticals, including ciprofloxacin, is usually based on results of standard laboratory toxicity experiments. Predictions based on such well-controlled conditions might deviate from the actual toxic effects in natural environments, due to various local environmental factors that influence pharmaceutical bioavailability. In other words, the fraction of pharmaceuticals in aquatic environments which is available for uptake by organisms is affected by real water chemistry factors.

For instance, pH can be a crucial factor regulating the fate of ionizable chemicals in aquatic environments (Alsop & Wilson, 2019; Bittner et al., 2019). Depending on pH, CIP can exist in five forms carrying different charges (Table 1), each with a different photodegradation fate (Babić et al., 2013; Salma et al., 2016). In terms of trans-membrane mobility, pH might affect the pathways by which CIP enters cells: the CIP zwitterion with zero net charge is more likely to penetrate the cell membrane via passive diffusion requiring zero energy, whereas charged ions might demand additional energy for active transport, and thus being less bioavailable (Ritchie & Islam, 2001; Xiong et al., 2021).

Table 1. Properties of the antibiotic ciprofloxacin (CIP).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Ciprofloxacin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Fluoroquinolones</td>
<td>-</td>
</tr>
<tr>
<td>CAS</td>
<td>85721-33-1</td>
<td>-</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>331.35 (CIP)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>367.82 (CIP.HCl)</td>
<td>-</td>
</tr>
<tr>
<td>Solubility</td>
<td>30 g L$^{-1}$ (20 °C, CIP.HCl)</td>
<td>Nowara et al. (1997)</td>
</tr>
<tr>
<td>log $K_{ow}$</td>
<td>0.28</td>
<td>Takács-Novák et al. (1992)</td>
</tr>
<tr>
<td>Acid dissociation constants</td>
<td>3.84, 5.05, 6.95, 8.95</td>
<td>Qiang &amp; Adams (2004)</td>
</tr>
</tbody>
</table>

In addition, dissolved organic matter (DOM) in waterbodies has also been related to the bioavailability of chemical pollutants (Chiou, 2003; Rizzuto et al., 2021). Dissolved organic carbon (DOC) can decrease the available concentration of pollutants by forming DOC-pollutant complexes, which may lead to reduced toxicity in fish and plankton (Black & McCarthy, 1988; Rowett et al., 2021).
2016). For CIP, both charged CIP\(^{\pm}\) (-1 ≤ x ≤ 3) and zwitterionic CIP\(^{\pm/}\) are potentially able to bind to DOC through various mechanisms, including electrostatic interactions, van der Waals, and multiple H-bonding interactions, with electrostatic interactions being the dominant mechanism for aquatic humic substances (Aristilde & Sposito, 2013; Carmosini & Lee, 2009). Such interactions between ionizable pharmaceuticals and DOM are possibly further regulated by divalent metal cations through the cation bridging effect and cation competition (Aristilde & Sposito, 2010; Carrasquillo et al., 2008; MacKay & Canterbury, 2005).

Given the above evidence, risk assessment of chemicals should take into consideration a variety of water chemistry factors, which however leads to a need for numerous laboratory experiments. To tackle this problem, mathematical models that predict the response of organisms to chemicals under different environmental conditions can be an efficient alternative, to reduce the number of experiments and to provide a tool for regulatory policy making (e.g., Escher et al., 2020; Van Sprang et al., 2016).

In this study, we investigated CIP ecotoxicity under the influence of three environmental factors, i.e. pH, dissolved organic carbon (DOC), and water hardness (calcium), by performing a cyanobacteria toxicity test with *Microcystis aeruginosa* PCC7806 according to the OECD guideline 201 (OECD, 2011) using cell growth rate as endpoint. Data collected from the toxicity experiments were analyzed to parameterize a bioavailability model predicting the relationship between the three environmental factors and CIP toxicity. We put forward four working hypotheses: (1) different CIP species have different toxicity, with CIP\(^{\pm/}\) being the most toxic; (2) CIP toxicity is negatively correlated with DOC because CIP-DOC binding reduces the available fraction of CIP; (3) the CIP-DOC binding process is regulated by pH indirectly via CIP speciation; and (4) the CIP-DOC binding process is also controlled by calcium (Ca\(^{2+}\)). Ca\(^{2+}\) competes with positively charged CIP species for DOC binding sites, but facilitates the binding between negatively charged CIP and DOC by a bridging effect.

2 Materials and methods
2.1 Cyanobacteria culture and test medium

The freshwater cyanobacteria *Microcystis aeruginosa* PCC7806 was obtained from the Pasteur Culture Collection of Cyanobacteria (PCC). *M. aeruginosa* was cultured in WC medium (Guillard & Lorenzen, 1972) at 24 ± 2 °C with 24-hour illumination of 27.0 ± 2.7 μmol m\(^{-2}\) s\(^{-1}\). The same conditions were employed for toxicity experiments.

In the toxicity tests, WC medium was modified to contain no Ca\(^{2+}\) (modified WC medium). Calcium chloride dihydrate (CaCl\(_2\cdot2\)H\(_2\)O, ACS reagent, 10035-04-8, Sigma-Aldrich USA) solution and Suwannee River natural organic matter (SRNOM, International Humic Substances Society), as well as dilute amounts from a 0.1 M HCl and a 0.1 M NaOH solution were applied to provide the desired combinations of pH, DOC and water hardness according to the experimental design (see below, Experimental design). No pH buffer was applied in toxicity experiments. pH was adjusted manually to desirable levels on a daily basis during exposure.
Ciprofloxacin hydrochloride (C₁₇H₁₈FN₃O₅·HCl·H₂O, CAS 86483-48-9, pharmaceutical EU standard grade) was purchased from Sigma-Aldrich (USA). A CIP stock solution (100 mg L⁻¹) was prepared by dissolving ciprofloxacin hydrochloride directly into modified WC medium. CIP stocks with lower concentrations were prepared from this stock solution by further diluting with modified WC medium. The concentrations of CIP stock solutions used in toxicity tests is listed in Table A1 in supplemental data. All stock solutions were stored at -20 °C and were defrosted in the dark at 4 °C, 24 hours before adding CIP to the test medium.

2.2 Cyanobacteria toxicity test

The cyanobacteria growth inhibition test was carried out in 50 mL culture medium in Erlenmeyer flasks and was slightly adapted compared with the OECD guideline (OECD, 2011). The duration of the toxicity test was extended to 96 hours to ensure a 16-fold increase in cell density. Inoculum cultures were prepared 4 days before the toxicity tests and cultured in the same conditions as in the toxicity test, in order to maintain *M. aeruginosa* within the exponential growth phase. During the test, flasks were shaken manually twice a day. The initial *M. aeruginosa* cell density in the toxicity test was 40,000 cell mL⁻¹. During the exposure, *M. aeruginosa* cell density was measured every 24 hours using a Beckmann Coulter Counter. The *M. aeruginosa* growth rate was calculated from daily recorded cell densities as endpoint.

2.3 Experimental design

Experiment I had pH (pH = 7.2, 7.8, 8.4, 8.8) as the sole environmental factor (Table 2). Experiment II included three independent variables: pH (7.0 to 9.0), dissolved organic carbon (0 to 24.0 mg L⁻¹), and water hardness (10 to 150 mg Ca²⁺ L⁻¹). For experiment II, a second order composite design (CCD) was applied to balance the potential detection of interactive effects between independent variables with limiting the number of tests (Lenth et al., 2018; Montgomery et al., 2009). The design was generated with the function *ccd* in RStudio Version 1.3.959 (R coreTeam, 2019), which gave 15 different combinations of pH, DOC, and Ca²⁺ (Table 3) classified into three groups: 8 cube points, 6 star points, and 1 center point. More detailed explanation about CCD and the classification of points can be found in Heijerick et al. (2003). Toxicity tests using the 15 media were arranged in four blocks, which were run separately but sequentially, with the ‘center’ medium (H1 – H6) repeated in every block. The concentrations of CIP tested in the two toxicity tests are listed in Table 2 (experiment I) and Table 3 (experiment II).

A light exposure experiment for CIP was carried out in 8 of the 15 media of the CCD design (Table A4 in supplemental data) in supplement of the toxicity tests, to evaluate possible photodegradation. Two concentration levels of CIP were added to each medium groups in triplicate and exposed to 27 ± 3 µmol m⁻² s⁻¹ lighting for 4 days, as in the toxicity tests. As for experiment I and II, *M. aeruginosa* was exposed to a gradient of CIP concentrations, with a 2-fold spacing factor between consecutive concentrations (Table 2 and 3).
2.4 Analytical chemistry

In the light exposure experiment, samples for CIP measurements were collected from each treatment every 24 hours. In a preliminary experiment, the influence of filtration was tested by collecting two types of samples: unfiltered and filtered (0.45 µm polyethersulfone (PES) membrane syringe filter) samples. During and at the end of the light exposure experiment, samples were collected without filtration.

In the toxicity tests, samples for Ca\(^{2+}\), DOC, and CIP measurements were collected at the beginning and at the end of the experiment. CIP samples were collected from every triplicate of the different treatments and merged together. Ca\(^{2+}\) and DOC samples were taken from the control replicates of each medium. Samples taken at the end of the tests were filtered with a 0.45 µm PES membrane filter. Ca\(^{2+}\) samples were acidified after sampling with nitric acid (final concentration = 0.1%) and stored at 4 °C. DOC samples were stored under the same condition. Analysis of dissolved Ca\(^{2+}\) concentration was performed with an Inductively Coupled Plasma-Mass Spectroscopy (ICAP 7000 Series). DOC samples were analyzed with a Total Organic Carbon Analyzer (Shimadzu TOC-L).

CIP samples collected from experiment II were stored at -20 °C. Prior to solid-phase extraction (SPE), Na\(_2\)EDTA.2H\(_2\)O (final concentration = 1.0 g L\(^{-1}\)) was added to 3.0 mL CIP samples, and the pH of the samples was adjusted to 3.0 ± 0.1 by addition of a 6.0 M NaOH solution. SPE was performed on Oasis HLB cartridges (6 mL, 200 mg sorbent). The cartridges were conditioned with 6.0 mL MeOH and 6.0 mL H\(_2\)O (HPLC grade) before 3.0 mL sample was loaded. The cartridges were washed with 18.0 mL H\(_2\)O (HPLC grade) and eluted with 5.0 mL MeOH/ACN (50/50, v/v %). The SPE extracts were evaporated under a gentle nitrogen flux until the liquid approached complete dryness, and were then reconstituted in 1.0 mL MeOH/water (10/90, v/v %) added with 0.1% (v/v %) formic acid and 0.1 g L\(^{-1}\) Na\(_2\)EDTA-2H\(_2\)O. Instrumental CIP 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), according to the method described in (Vanryckeghem et al., 2019). Calibration and quantification are described in Section S2 in in supplemental data. We considered a deviation of 30% or less between the nominal CIP concentration and the concentrations measured before the experiments as acceptable. The difference in CIP concentrations measured at the start and the end of experiments is defined as the ‘loss’, expressed in percentage relative to the CIP concentration measured at the start of experiments.

2.5 Statistical analysis

All analysis were performed with RStudio Version 1.3.959 (R coreTeam, 2019).

Cell growth rate was calculated based on *M. aeruginosa* cell density transformed into a cell growth rate (Eq. 1).

\[
\text{r}_{Gr} = \frac{\ln(\text{CD}_{t+1}) - \ln(\text{CD}_{t})}{t_{t+1} - t_{t}} \quad \text{Eq. 1}
\]
where $r_G$ is the 24-hour growth rate of *M. aeruginosa* (per day) of the $i^{th}$ day, $CD_i$ and $CD_{i+1}$ are the cell density of *M. aeruginosa* measured at time $t_i$ and $t_{i+1}$, respectively. A TukeyHSD test was performed to compare the cell growth rate in the control group of different media. The medium whose control group was identified by TukeyHSD to have cell growth rates significantly slower than that in the control groups of other media were excluded from further data interpretation.

Calculation of the dose-response relationship employed the overall cell growth rate ($r_{Gr,treatment}$).

Daily measurements of cell density were ln-transformed and plotted against time. $r_{Gr,treatment}$ is defined as the slope of a linear regression line drawn for ln-transformed cell density as a function of time. The 96 hour median effect concentration (96h EC50) of CIP was obtained using a 3-parameter log-logistic dose-response model (Ritz et al., 2016) based on $r_{Gr,treatment}$ and nominal CIP concentration (Eq. 2). The 96h EC50 is also expressed as the observed overall CIP median effect concentration in a specific environmental condition, EC50$_{total}$.

$$r_{Gr,treatment} = \frac{r_{Gr,max}}{1+(\frac{[CIP]}{50} \text{EC50}_t)}$$  \hspace{1cm} \text{Eq. 2}

in which $r_G$ is the observed growth rate in experiments (per day), $r_{Gr,max}$ is the maximum growth rate of *M. aeruginosa* (per day), [CIP] is the concentration of CIP in the exposure vessel, and $s$ is the slope factor (-) in the dose-response relationship. Negative growth rate (fatality) was excluded from the analysis of 96h EC50. We used the mean of measured values of independent variables for statistical analysis.

### 2.6 Bioavailability model: a concentration additive effect model

The model established to simulate CIP bioavailability to *M. aeruginosa* and CIP’s influence on *M. aeruginosa* growth rate is a 0-dimensional model without spatial structure. Environmental conditions (pH, DOC and Ca$^{2+}$ levels) are treated as external variables. The model was built with Rstudio using package `deSolve` (Soetaert et al., 2010) with two compartments:

![Figure 1. Simplified schematic structure of the equilibrium module in the ciprofloxacin (CIP) bioavailability model.](image)

*CIP$_{total}$* is the amount (mole) of CIP added into the system. The equilibrium model in the blue circle is the key section that calculates EC50$_{total}$, the overall median effect concentration of CIP as model output. CIP$^+$, CIP$^{+/−}$, CIP$^{−}$ are the concentrations of the three CIP species. DOC-CIP$^+$ and DOC-CIP$^{+/−}$ represent the CIP$^+$ and CIP$^{+/−}$, respectively, that is bound to DOC. $pK_a$ and $pK_{na}$ are the third and fourth acid dissociation constants of CIP; $K_{doc}^+$ and $K_{doc}^{+/−}$ are the binding constants for DOC-CIP$^+$ and DOC-CIP$^{+/−}$.
(1) an equilibrium module simulates the speciation of CIP under the influence of pH and DOC, giving EC50_total as the output (Figure 1). Ca^{2+} was removed from the model after data analysis.

(2) a detachable module simulates changes in the two state variables, i.e., *M. aeruginosa* cell (cells) and the amount of CIP (mole), with differential equations. This is to allow applying the bioavailability model for species other than *M. aeruginosa*.

The key element emphasized by this study is the dynamic balance model in the first compartment. In it, speciation is calculated based on the Henderson–Hasselbalch equation. In the tested pH range (7.0 to 9.0), CIP^+, CIP^{+/-}, CIP^- are the most relevant CIP species. Their relationship is given in Eq. 3 and Eq. 4.

\[
\text{pH} = pK_{a3} + \log_{10} \frac{[\text{CIP}^{+/-}]}{[\text{CIP}^+]} \quad \text{Eq. 3}
\]

\[
\text{pH} = pK_{a4} + \log_{10} \frac{[\text{CIP}^-]}{[\text{CIP}^{+/-}]} \quad \text{Eq. 4}
\]

in which pK_{a3} and pK_{a4} are the 3\text{rd} and 4\text{th} acid dissociation constant of CIP (Qiang & Adams (2004). It is assumed that species of CIP reach an instant equilibrium in the aqueous environment. The interaction between DOC and CIP is simulated as a binding process (Eq. 5).

\[
[\text{DOC} - \text{CIP}] = [\text{DOC}] \times (K_{\text{doc}}^{+} \times [\text{CIP}^+] + K_{\text{doc}}^{+/-} \times [\text{CIP}^{+/-}] + K_{\text{doc}}^{-} \times [\text{CIP}^-]) \quad \text{Eq. 5}
\]

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

In the second compartment, the model assumes that CIP toxicity effects can directly reflect on *M. aeruginosa* growth. The overall EC50 value is calculated based on the additive effect concentration model, assuming that DOC–CIP is not toxic (Neuwoehner & Escher, 2011; Sun et al., 2020).

\[
\frac{1}{\text{EC50}_{\text{total}}} = \frac{f_{\text{CIP}^+}}{\text{EC50}(\text{CIP}^+)} + \frac{f_{\text{CIP}^{+/-}}}{\text{EC50}(\text{CIP}^{+/-})} + \frac{f_{\text{CIP}^-}}{\text{EC50}(\text{CIP}^-)} \quad \text{Eq. 6}
\]

where EC50_{total} is the total median effect concentration in a specific environment (i.e., the sum of concentrations of all CIP species at 50% effect), f_{CIP^+} equals [CIP^+]/[CIP_{total}], f_{CIP^-} equals [CIP^-]/[CIP_{total}], f_{CIP^{+/-}} equals [CIP^{+/-}]/[CIP_{total}], EC50(CIP^+), EC50(CIP^-), EC50(CIP^{+/-}) are the median effect concentration of CIP^+, CIP^-, and CIP^{+/-}, respectively.

### 2.7 Parameterization

The bioavailability model was fitted to experimental toxicity results to calculate the best value for model parameters using ‘optim’ in RStudio (Ghalanos et al., 2012). Six parameters, K_{doc}^+, K_{doc}^{+/-}, K_{doc}^-, and EC50(CIP^+), EC50(CIP^{+/-}), EC50(CIP^-) were parameterized using a 2-step strategy. First, three EC50s from Eq. 6 were parameterized with results collected from experiment I. Secondly, the
EC50s obtained in the first step were applied when parameterizing Kdoc\(^-\), Kdoc\(^+/\) and Kdoc\(^+\), using the experimental results from experiment II.

Model performance was evaluated in terms of the sum of squared errors (SSE) between ln-transformed predicted EC50\(_{\text{total}}\) and ln-transformed observed EC50\(_{\text{total}}\) (Section S4 in supplemental data). In addition, the Pearson’s correlation analysis was performed between EC50\(_{\text{total}}\) predictions and observations. The parameter set with the lowest SSE and the strongest Pearson correlation was determined as the most suitable one.

### 3 Results

#### 3.1 Water chemistry

In experiment I, pH was controlled within the nominal pH level ± 0.2. DOC was below the quantification limit (LOQ, 0.92 mg L\(^-1\)), while the Ca\(^{2+}\) concentration was on average between 6.5 and 7.1 mg L\(^-1\) (Table 2). In experiment II, the mean pH deviated from the nominal pH within ± 0.3 (Table 3). Temperature was controlled within 24.0 ± 2.0 °C. The DOC concentration in toxicity experiment II varied between 0 and 20.4 mg L\(^-1\). Measured DOC was within ± 0.8 mg L\(^-1\) of the nominal levels.

Ca\(^{2+}\) concentration ranged from 5.6 to 152.4 mg L\(^-1\). In media L, M, N, and H6, the measured Ca\(^{2+}\) concentration was only half of the nominal level due to a spiking error made by the experimenter. This was confirmed in the laboratory notes of the experimenter, and was not due to measurement error or Ca precipitation.

In the CIP samples taken during the light exposure experiments, the deviations between CIP concentration in samples collected on day 0 and the nominal CIP concentration were all within ± 30%. Only in 1 out of the 16 media, CIP showed a CIP loss above 20% (Table A6). The CIP degradation rate in the light exposure experiment is calculated based on the assumption of a 1\(^{\text{st}}\) order degradation process as shown by Eq. 7.

\[
\frac{d[CIP_{\text{total}}]}{dt} = -r_{\text{Deg,CIP}} \times [CIP_{\text{total}}] \tag{7}
\]

where [CIP\(_{\text{total}}\)] is the total amount of CIP in the system, \(r_{\text{Deg,CIP}}\) is the first order degradation rate constant of CIP, and \(\frac{d[CIP_{\text{total}}]}{dt}\) is the rate of change in the total amount of CIP. Degradation rate constants were between 0.001 (pH 7.0) and 0.078 (pH 9.0) day\(^-1\) (Table A6) across all test media and presented a significant positive correlation with pH (Figure A4). Mathematically, the mean CIP loss due to degradation in a 96-hour experiment in our test condition is 10%. The impact caused by degradation was considered limited and, therefore, we decided to omit CIP degradation in our data analysis (OECD, 2011). Considering the limited impact of degradation, we also decided to mute the degradation function in the bioavailability model (\(r_{\text{Deg,CIP}} = 0\)), allowing the equilibrium module along to predict CIP toxicity.

In CIP samples taken from experiment II, 92.5% of samples taken on day 0 had deviations below 30% (Table A7). The CIP concentration in the two samples selected from Medium E deviated from the nominal concentration by 35% and 38%, respectively. Loss of CIP during experiment II was
between 0 to 53\% (results with deviation \geq 30\% were excluded from analysis), with 75\% of the loss values being higher than 20\% and a mean loss of 30\%. Given that measured CIP concentrations mostly agree with the nominal concentrations, the nominal CIP concentration was used to represent the CIP concentration to which \textit{M. aeruginosa} was exposed during experiment I and II in all following data analysis.
Table 2. The nominal and measured values of water chemistry factors with standard deviation: dissolved organic carbon (DOC, n=2), calcium (Ca$^{2+}$, n=2), pH (n=5), and the 96-hour median effect concentration (96h EC50) of ciprofloxacin (CIP) in experiment I with 95% confidence interval. The lower quantification limit (LOQ) of DOC is 0.92 mg L$^{-1}$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Medium</th>
<th>Nominal pH (-)</th>
<th>Nominal DOC (mg L$^{-1}$)</th>
<th>Nominal Ca$^{2+}$ (mg L$^{-1}$)</th>
<th>Nominal CIP range (µg L$^{-1}$)</th>
<th>Measured pH (-)</th>
<th>Measured DOC (mg L$^{-1}$)</th>
<th>Measured Ca$^{2+}$ (mg L$^{-1}$)</th>
<th>96h EC50 (µg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-a</td>
<td>a</td>
<td>7.2</td>
<td>0.90 - 3.60</td>
<td>7.35</td>
<td>0.90 - 3.60</td>
<td>7.2 ± 0.2</td>
<td>6.5 ± 0.0</td>
<td>1.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>7.8</td>
<td>0</td>
<td>0.68 - 2.70</td>
<td>7.9 ± 0.2</td>
<td>&lt;LOQ</td>
<td>6.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>8.4</td>
<td>0.68 - 2.70</td>
<td>8.4 ± 0.1</td>
<td>7.0 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>8.8</td>
<td>0.90 - 3.60</td>
<td>8.8 ± 0.2</td>
<td>7.0 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>7.2</td>
<td>0.18 - 2.88</td>
<td>7.3 ± 0.1</td>
<td>7.1 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>f</td>
<td>7.9</td>
<td>0.18 - 2.88</td>
<td>7.9 ± 0.1</td>
<td>7.1 ± 0.4</td>
<td>0.9 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>g</td>
<td>8.6</td>
<td>0.18 - 2.88</td>
<td>8.6 ± 0.1</td>
<td>7.1 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>h</td>
<td>9.3</td>
<td>0.18 - 2.88</td>
<td>9.2 ± 0.1</td>
<td>7.1 ± 0.4</td>
<td>1.3 ± 0.1</td>
<td></td>
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</table>
Table 3. The nominal and measured values of water chemistry factors with standard deviation: dissolved organic carbon (DOC, n=6), calcium (Ca\(^{2+}\), n=6), pH (n=5), and the 96-hour median effect concentration (96h EC50) of ciprofloxacin (CIP) in experiment II with 95% confidence interval. The lower quantification limit (LOQ) of DOC is 0.92 mg L\(^{-1}\).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Nominal pH (-)</th>
<th>Nominal DOC (mg L(^{-1}))</th>
<th>Nominal Ca(^{2+}) (mg L(^{-1}))</th>
<th>Nominal CIP range (µg L(^{-1}))</th>
<th>Group</th>
<th>Block</th>
<th>Measured pH (-)</th>
<th>Measured DOC (mg L(^{-1}))</th>
<th>Measured Ca(^{2+}) (mg L(^{-1}))</th>
<th>96h EC50 (µg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.6</td>
<td>4.9</td>
<td>121.6</td>
<td>0.36 - 2.88</td>
<td>Cube</td>
<td>1</td>
<td>8.5 ± 0.1</td>
<td>6.5 ± 1.2</td>
<td>111.0 ± 2.3</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>7.4</td>
<td>4.9</td>
<td>38.4</td>
<td>0.54 - 4.32</td>
<td>Cube</td>
<td>1</td>
<td>7.5 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>36.1 ± 0.8</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>C</td>
<td>7.4</td>
<td>19.1</td>
<td>121.6</td>
<td>1.62 - 13.0</td>
<td>Cube</td>
<td>1</td>
<td>7.5 ± 0.2</td>
<td>18.6 ± 0.3</td>
<td>112.1 ± 2.5</td>
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<td>D</td>
<td>8.6</td>
<td>19.1</td>
<td>38.4</td>
<td>0.45 - 3.60</td>
<td>Cube</td>
<td>1</td>
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<td>17.2 ± 0.5</td>
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<td>8.0 ± 0.2</td>
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<td>Cube</td>
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<td>1.35 - 10.8</td>
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<td>0.68 - 5.41</td>
<td>Star</td>
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<td>10.7 ± 0.4</td>
<td>42.1 ± 0.3</td>
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</table>
3.2 Ciprofloxacin toxicity under different water chemistry conditions

In experiment I, the 96-hour EC50 (96h EC50) values across pH 7.2 to 9.3 were between 0.7 and 1.3 µg L\(^{-1}\) (Figure 2). When pH was varying from neutral to alkaline conditions, the EC50 observations first dropped but rose again after pH 7.8, suggesting that the strongest toxic response was triggered at pH 7.8.

![Figure 2](image)

Figure 2. 3-parameter logistic dose-response curves of ciprofloxacin (CIP) in experiment I-a (a-d, crosses) and I-b (e-h, circles). Letter a-h each represent a test medium. The mean pH of test media is shown on the corresponding plot. Data of the highest CIP concentration level in medium f, g, h were excluded from analysis because they showed negative cell growth rates.

In experiment II, multiple independent variables contributed to a 7-fold variation in the 96h EC50s to *M. aeruginosa* across different media, i.e., from 1.1 to 7.0 µg L\(^{-1}\) (Figure 3). The medium with the highest 96h EC50, medium O, was the medium with the lowest pH level. Medium O also showed a relatively slow *M. aeruginosa* cell growth rate in comparison to all other media. Wilcoxon rank sum test revealed that the cell growth rate in the control group of medium O is significantly lower than that in all other media (Table A8). By removing this outlier, the variation in the 96-hour EC50s decreased to 4-fold, i.e. between 1.1 and 4.6 µg L\(^{-1}\).
Figure 3. 3-parameter logistic dose-response curves of ciprofloxacin (CIP) in the 20 media of experiment II. One point in the reference group of medium C was removed because cells did not grow in that culture. Letter A-O each represent a test medium. The water chemistry factors: pH (+), DOC (mg L⁻¹), and Ca (mg L⁻¹), of test media are shown on the corresponding plot. The highest CIP level in medium K was removed because it caused a negative cell growth rate. The highest CIP level in medium H6 was also removed because the cell growth rate at that treatment was significantly different from that in the other ‘center’ media.

3.3 CIP bioavailability model

Parameterization was first performed for the EC50 of CIP⁺, CIP⁻/⁺ and CIP⁻, with data collected from experiment I. The EC50 of CIP⁻/⁺ was found to be 653 ng L⁻¹ (1.97 nmol L⁻¹), while the EC50 of CIP⁺ and CIP⁻ were set to 10¹² nmol L⁻¹, as they tended to be infinite (i.e. not contributing to toxicity) (Table 4). In other words, CIP⁺ and CIP⁻ are considered to be not toxic. This allows us to simplify Eq. 6 to Eq. 8.
EC50(CIP+/−) = EC50_{total} x fCIP+/−  \tag{eq. 8}

In the second step, parameterization used data collected from experiment II and above results (i.e. EC50(CIP+/−) = 653 ng L⁻¹, EC50(CIP+) = EC50(CIP−) = 3.3 x 10¹⁴ ng L⁻¹ (10¹² nmol L⁻¹) as input; 3.3 x 10¹⁴ ng L⁻¹ (10¹² nmol L⁻¹) was employed to represent infinite). The results of medium O as an outlier were excluded from analysis. Parameterization results suggest that CIP⁺, CIP⁺⁺ and CIP− all take part in CIP-DOC interactions (Table 4). CIP⁺ and CIP⁺⁺ play an important role in the alteration of EC50_{total}, with K_{doc}⁺ (177 L g⁻¹) and K_{doc}⁺⁺ (273 L g⁻¹), compared to CIP− (K_{doc}− = 0 L g⁻¹).

Table 4. Estimate of parameter values in the bioavailability model using the ‘optim’ function in RStudio. SSEₚ₀ = \sum_i^[\ln(\text{EC50}_{\text{total, predicted}}) - \ln(\text{EC50}_{\text{total, observed}})]^2. The result of medium O was removed from analysis because the mean cell growth rate of the control group in it was significantly lower than in other media. 3.3 x 10¹⁴ was applied in parameterization to simulate ‘infinite’.

<table>
<thead>
<tr>
<th>EC50 (ng L⁻¹)</th>
<th>K_{doc}⁺⁺</th>
<th>K_{doc}⁺</th>
<th>K_{doc}−</th>
<th>SSEₚ₀</th>
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</thead>
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<tr>
<td>3.3 x 10¹⁴</td>
<td>653</td>
<td>3.3 x 10¹⁴</td>
<td>273</td>
<td>177</td>
</tr>
<tr>
<td>3.3 x 10¹⁴</td>
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<tr>
<td>3.3 x 10¹⁴</td>
<td>653</td>
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4 Discussion

We investigated the response of *M. aeruginosa* to CIP in a variety of test media, with different pH, DOC, and Ca levels. pH and DOC, two water chemistry properties that can vary from one waterbody to another, were identified as key regulators of CIP toxicity. As traditional ecotoxicity tests are often performed at a single fixed pH level and in the absence of added organic matter, the toxicity of ionizable pharmaceuticals like CIP estimated from laboratory tests can be mismatched with toxicity in the field. In what follows, we further discuss the linkage between CIP toxicity and these environmental factors in terms of bioavailability as well as other interesting observations related to CIP toxicity that we observed during our experiments.

CIP degradation during the experiments reduced the concentration available to *M. aeruginosa*, leading to the nominal EC50 overestimating the real EC50. Yet, the CIP loss observed in the light exposure experiment, as well as the theoretical maximum CIP loss during a 4-day exposure calculated from the first order degradation rate, was never more than 20%. Compared to the light exposure experiment, the CIP loss in the toxicity tests was on average higher than 20% (Table A7), suggesting that the additional CIP loss was primarily due to uptake, adsorption or absorption by the organisms (Subashchandrabose et al., 2013; Wright et al., 1977).

In fact, the CIP degradation under light exposure observed in our test was 3 to 4 orders of magnitude slower than that reported in other photodegradation studies. Wei et al. (2013) reported CIP photolytic rate constants between 1.2 to 1.5 hour⁻¹ between pH 7 and 9 (30 to 36 day⁻¹) under simulated sunlight (Xenon lamp, at 9 µmol m⁻² s⁻¹). A degradation experiment by Sturini et al. (2012) was conducted under natural sunlight (95-154 µmol m⁻² s⁻¹ visual light and 6-10 µmol m⁻² s⁻¹ ultraviolet light), from which a first order photodegradation rate constant as high as 0.22 minute⁻¹ (317 day⁻¹) was
obtained. Besides potential differences in experimental conditions like pH, light wavelength and intensity (Gad-Allah et al., 2011; Wei et al., 2013), the composition of our test medium, an artificial freshwater, might have contributed to slowing down the overall degradation. Various ions like Fe\(^{3+}\), NO\(_3\)^-, Cl\(^-\), as well as humic substances (HS) can reduce the abundance of ROS and therefore inhibit photo-induced degradation reactions (Babić et al., 2013; Ge et al., 2010).

The EC50 for CIP to *M. aeruginosa* reported by other studies is usually within the same order of magnitude as in the present study. Halling-Sørensen et al. (2000) reported an EC50 of CIP to *M. aeruginosa* between 4 and 6 µg L\(^{-1}\) in a standard toxicity test (OECD, 1984). The value reported by Liu et al. (2017) is between 3.40-3.83 µg L\(^{-1}\) (15 days, 25 ± 1.0 °C). Azevedo et al. (2019) found a CIP EC50 up to 206 µg L\(^{-1}\) (4 days, 25 ± 2.0 °C) in their toxicity test conducted at neutral pH and using an initial cell density (4 x 10\(^6\) cell mL\(^{-1}\)) which is 100-fold higher than in our study (4 x 10\(^4\) cell mL\(^{-1}\)). Despite these differences, in our experiments, medium O with neutral pH also showed an average cell growth rate (0.58 day\(^{-1}\)) that was significantly lower (TukeyHSD) than the other media. This medium showed the highest 96 h EC50, 7.0 µg L\(^{-1}\), which is also 30% higher than the EC50\(_{\text{total}}\) predicted by our bioavailability model (4.9 µg L\(^{-1}\)). This observation, together with that of Azevedo et al. (2019) hint a possible linkage between pH, cell growth rate, and CIP toxicity, which is discussed later in this paper.

When CIP\(^{\text{H}^+}\) would be the only toxic species, the highest CIP toxicity is expected where CIP\(^{\text{H}^+}\) is most abundant, which is when pH is the mean of CIP’s pK\(_{\text{a1}}\) and pK\(_{\text{a2}}\) values (i.e., at pH 7.95). In experiment I, we observed that the CIP EC50\(_{\text{total}}\) decreased from 1.1-1.2 µg L\(^{-1}\) at around pH 7.2 to 0.7-0.9 µg L\(^{-1}\) at pH 7.9, followed by an increase to 1.3 µg L\(^{-1}\) at pH 9.2 (Figure 4, open and closed circles). At the lower end of this pH range (pH 7.2) CIP\(^+\) and CIP\(^{\text{H}^+}\) account for around 35.5% and 63.1% of the total CIP (Figure A2 in supplemental data), respectively, whereas at pH 7.9, 83.2% of CIP is in the form of CIP\(^{\text{H}^+}\). At the higher end (pH 9.2), the fraction of CIP\(^{\text{H}^+}\) in total CIP drops to 35.9% and 63.8% of total CIP is CIP. Although the overall variation in CIP EC50\(_{\text{total}}\) in experiment I was relatively small (the highest EC50\(_{\text{total}}\) < 2-fold the minimum EC50\(_{\text{total}}\)), it is clear that CIP toxicity

![Figure 4. Predicted EC50\(_{\text{total}}\) (solid pink line) of ciprofloxacin (CIP), observed EC50\(_{\text{total}}\) in experiment I-a (closed circles) and I-b (open circles) of CIP, the theoretical fraction of CIP\(^{\text{H}^+}\) in total CIP amount (fCIP\(^{\text{H}^+}\), orange dashed line), and the product of EC50\(_{\text{total}}\) x fCIP\(^{\text{H}^+}\) in experiment I-a (closed triangle) and I-b (open triangle). The black vertical lines on the circles show the 95% confidence interval of EC50, and the purple horizontal lines indicate the standard error in pH. EC50(CIP\(^{\text{H}^+}\)) obtained by parameterization is shown by the green dotdashed line.](image-url)
was lower when less CIP\textsuperscript{+/-} was present and that it was higher when there was more CIP\textsuperscript{+/-}, which supports our initial hypothesis (please see introduction).

However, EC50\textsubscript{total} observations do not fully echo with the model-predicted EC50\textsubscript{total} (Figure 4, pink solid line) across the whole pH range. Especially at pH 8.8 and 9.2, the predictions are underestimates, possibly due to variations in pH during the toxicity experiment. Because no suitable pH buffer can be applied in this study, pH values in the cultures with nominal pH above 8.5 are challenging to maintain, presenting a strong tendency to decrease its pH. Compared to the mean pH applied in data analysis, the median pH might better represent the true environment to which the cells were exposed, but continuous monitoring of pH was impossible during the experiments. Following Eq. 8, the product of EC50\textsubscript{total} and the fraction of CIP\textsuperscript{+/-} in CIP\textsubscript{total} (closed and open triangles in Figure 4) is expected to equal the EC50 of CIP\textsuperscript{+/-} (Figure 4, green dot-dashed line) at all pH levels. An overestimation of pH at the alkaline end could lead to underestimated EC50\textsubscript{total}.

On the opposite end of pH, an additional mechanism relating pH, cell growth rate, and CIP uptake is suspected. Cyanobacteria have pH preference. For several \textit{M. aeruginosa} strains, the suitable pH range is above 7.0. McLachlan and Gorham (1962) observed a sharp decline in \textit{M. aeruginosa} NCR-1 growth rate when pH dropped from pH 7.0 to pH 6.0. After 8-day incubation, the observed cell density at pH 6 was only one seventh than that at pH 7. Van der Westhuizen and Eloff (1985) reported a decrease in \textit{M. aeruginosa} UV-006 growth rate from 0.6 to 0.2 day\textsuperscript{-1} when pH dropped from 9.0 to 6.5. Our study witnessed the growth of \textit{M. aeruginosa} PCC7806 at pH 7.1 being significantly slower than that at higher pH levels between 7.5 and 8.8 (Table A8 in supplemental data), a pH range allowing \textit{M. aeruginosa} to grow at comparable rates.

When pH is close to 7, nutrient availability and the relatively H\textsuperscript{+} rich environment might hinder \textit{M. aeruginosa} cell growth. Bicarbonate, the vital material for primary production, becomes less abundant at pH 7 compared to pH 8. Thus, pH might direct cyanobacteria to invest more energy in inorganic carbon uptake than in replication (Mangan et al., 2016). The relatively high H\textsuperscript{+} content, on the other side, can also affect the surface binding sites on the \textit{M. aeruginosa} cell wall and trigger the cells to alert their membrane components (Guan & Liu, 2020; Singh et al., 2002). Trans-membrane transport, including the penetration of CIP into cells, might be affected negatively. As a result, physiological changes of cyanobacteria metabolism at neutral pH might partially account for the higher-than-expected 96h EC50 observed in medium O.

Compared to pH, the role of DOC is more straightforward, expressed by a positive correlation (R = 0.89, n = 19) between DOC and experimentally determined 96h EC50 values (Figure 5). When DOC was present in the system, binding to DOC decreased the free, bioavailable fraction of CIP to \textit{M. aeruginosa}, leading to a rise in EC50 with increasing DOC concentrations. However, while binding with DOC rose the overall EC50 by 4-fold in our experiments, the EC50 levels remain within the same order of magnitude.
Figure 5. Correlation between the 96-hour median effect concentration of ciprofloxacin (CIP) (96h EC50) in test media and (A) DOC (mg L$^{-1}$), (B) Ca$^{2+}$ (mg L$^{-1}$) and (C) pH (-), analysed with Pearson’s correlation. Results of medium O were excluded from analysis because the _M. aeruginosa_ cell growth rate in the control group of medium O was significantly lower than that in other media.

DOC as a regulator of CIP toxicity is partially dependent on pH. Assembling all the findings above mathematically, CIP toxicity in a specific environment influenced by DOC and pH (limited to pH 7 to 9) can be described by Eq. 9:

$$EC_{50_{total}} = \frac{EC_{50_{CIP+/−}}}{1 + 10^{6Kd_m-pH_2 + \phi (1+([DOC] x K_{doc^+})^+ + 10^{6Kd_m-pKa_4 + [DOC] x K_{doc^+/−}} \quad \text{Eq. 9}$$

The equation expresses that CIP$^{+/-}$ is the only toxic CIP species, and that the fraction of CIP$^{+/-}$ is regulated by pH and the sorption of different CIP species to DOC. Interestingly, parameterization results with similar low SSE$_{in}$ (Table 4, Table A11) seem to suggest that $K_{doc^{+/-}}$ is the most vital player. When both $K_{doc^{+}}$ and $K_{doc^{-}}$ are set at zero, the predicted $EC_{50_{total}}$ still agrees well with $EC_{50_{total}}$ observations ($R = 0.92$, n =19). Setting $K_{doc^{+/-}}$ to zero, on the other hand, leads to poorer predictions ($R < 0.75$, n =19). The limited pH range (7.1-8.8) tested in experiment II could partially explain why the contribution of CIP$^{+/-}$ to CIP-DOC interaction is most pronounced, as CIP$^{+/-}$ always accounted for more than 50% of free CIP ions in this range (Table A2).

In nature, the various types of DOC possess different binding capacities, affected by their composition (Carmosini & Lee, 2009; Haftka et al., 2010; Vitale & Di Guardo, 2019). For instance, even though humic substances (HS) from the same source as in our study (SRNOM) was employed by Aristilde and Sposito (2013), they found that the binding (described by the Bemmelen-Freundlich isotherm, Eq. 10) of CIP to HS was several times higher than that identified in our work.

$$Q = K_{OC}([CIP]_{eq})^\beta \quad \text{Eq. 10}$$

in which $Q$ is the adsorption capacity (mol g$^{-1}$), $K_{OC}$ is the Freundlich coefficient normalized to the carbon content of HS (mol L$^{-1}$), $[CIP]_{eq}$ is the aqueous CIP concentration at equilibrium (L g$^{-1}$), and $\beta$ is a slope parameter (-) close to 1. Consequently, CIP adsorption in Eq. 10 approximates a linear process, which allows us to compare carefully the $K_{OC}$ with the binding constant determined in this study. $K_{OC}$ of CIP to humic acid (HA) and fulvic acid (FA) were around 1600 L g$^{-1}$ and 1000 L g$^{-1}$, respectively, i.e., six and four times the binding constant of DOC (as a mixture of HA, FA, and other organic substances) in our study (273 L g$^{-1}$). Figure 6A translates variations in $K_{doc^+}$ or $K_{doc^{+/-}}$ to
CIP EC50<sub>total</sub> using our bioavailability model. When K<sub>doc</sub><sup>+</sup> and K<sub>doc</sub><sup>+/−</sup> changes from 0 to 3000 L g<sup>−1</sup>, predicted EC50<sub>total</sub> increases by 4 to 14-fold.

![Graph](image)

Despite the variation in sources of DOM and/or binding constants across studies, the bioavailability model provides an advantage that no traditional laboratory experiments can offer: simulating CIP toxicity across various environmental conditions. The binding of antibiotics to DOC is under dynamic balance, giving DOC the potential to become both a ‘collector’ and a ‘distributor’ of antibiotics depending on water chemistry conditions. Binding to DOC also prevents CIP from photodegradation (Belden et al., 2007), and can prolong the retention time of CIP in the environment. Once the equilibrium is disturbed, CIP can be released from DOC which increases its bioavailability again. In Yangtze river, for example, a positive relationship was identified between antibiotics concentrations and DOC concentrations (Yan et al., 2013), indicating that DOC was rather a source of antibiotics in that environment. As climate change is altering the abundance and magnitude of DOC in aquatic ecosystems (Creed et al., 2018), it is becoming increasingly important that policy makers can apply a suitable tool, like a bioavailability model, to simulate the dynamic influence of DOC on pharmaceutical ecotoxicity in nature.

In terms of the impact of Ca<sup>2+</sup> on CIP–DOC binding, no observations indicate effects such as cation competition (Aristilde & Sposito, 2010) or cation bridging that facilitates the binding of CIP to DOC (Aristilde & Sposito, 2013; MacKay & Canterbury, 2005). Cultures with high Ca<sup>2+</sup> concentration did not show an observable drop in CIP EC50<sub>total</sub>, which means that cation exchange is unlikely a significant contributor to CIP–DOC binding. The fact that K<sub>doc</sub><sup>−</sup> = 0 L g<sup>−1</sup> attributes CIP–DOC binding to the positively charged amine group on CIP, suggesting that electrostatic interaction is the main force between CIP and DOC in the tested pH range. Regarding the absence of cation bridging effect, the relatively unstable complexes formed by earth metals and fluoroquinolones is maybe a limiting factor (Cuprys et al., 2018; Zhang et al., 2012).

Overall, based on the impact of pH and DOC, our estimation of CIP EC50<sub>total</sub> to M. aeruginosa (Figure 6) is at least 10-fold higher than the CIP concentrations observed in European surface waters,
ranging between 1 and 100 ng L\(^{-1}\) (Kovalakova et al., 2020). Cyanobacteria are the species most sensitive to CIP among aquatic organisms (Ebert et al., 2011; Robinson et al., 2005). The current environmental quality standard (EQS) of CIP under the Water Framework Directive (WFD), 0.089 μg L\(^{-1}\) (Loos et al., 2018), is determined based on toxicity data of cyanobacteria Anabaena flos-aquae. M. aeruginosa shows higher sensitivity to CIP than the Anabaena, albeit it is protected by the current regulations. The 96h EC10 observed in medium K, 0.4 μg L\(^{-1}\) (Table A3), is 4 times higher than the current EQS. As medium K has no DOC addition and a mean pH around 7.9, it allows CIP to exist mostly in free CIP\(^{+\cdot}\) form (83.2%), which corresponds to the highest toxicity expected. In the field, with variable pH and the presence of DOC above 0 mg/L, the EC10 expectation is therefore equal to or above 0.4 μg L\(^{-1}\). Thus, the current EQS is likely a protective figure to the primary producer community in European waterbodies.

Nonetheless, the potential of CIP to generate antimicrobial resistance (AMR) deserves attention (Ng & Gin, 2019; Taylor et al., 2011). What does not kill bacteria might make them stronger. The sublethal effects caused by antibiotics in the environment can select bacteria strains that developed anti-microbial resistance (Gullberg et al., 2011; Sinel et al., 2017). Thus, the bioavailability model may also be a tool to simulate the effect of pH and DOC pressure faced by bacteria for studies focusing on AMR, especially given that both environmental factors, pH and DOC, are site-specific characteristics varying from water body to water body. Combining the bioavailability model with a model predicting the properties of organic matter, or with a geographic database to provide water chemistry properties, will extend the applicability of our bioavailability model.

5 Conclusions
Our study supports the hypothesis that CIP\(^{+\cdot}\), with zero net charge is the most bioavailable CIP species to M. aeruginosa, making the CIP\(^{+\cdot}\) concentration a better predictor for the chemical’s toxic effects than the total dissolved CIP concentration. Furthermore, DOC and pH were identified as the dominant regulators for CIP bioavailability, while water hardness represented by Ca\(^{2+}\) concentrations showed insignificant impact. The key, linking CIP bioavailability and its toxicity, is a combination of CIP-DOC binding and pH-dependent speciation. Our experiments covered around 90% of the environmental factors (pH, DOC and Ca\(^{2+}\)) in EU surface water. Within this range, a 7-fold effect on CIP toxicity is observed. As M. aeruginosa is a species relatively sensitive to CIP, the current EQS under the EU WFD is a protective figure for the species, and likely sufficient to protect aquatic ecosystems.

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Interactions of the herbicide propanil and a metabolite, 3,4-dichloroaniline, with blue-green algae

