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Binding of waterborne pharmaceutical and personal care products to natural dissolved organic matter

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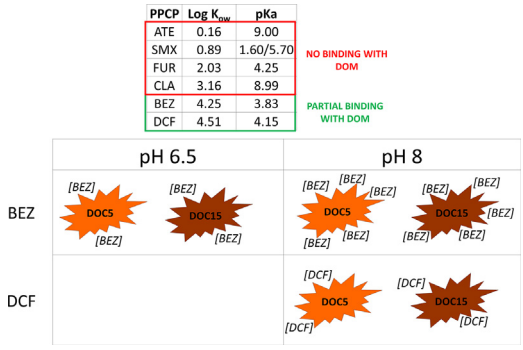
HIGHLIGHTS

- Natural DOM partially bind to PPCPs with carboxylic functional groups.
- DOM-PPCPs binding dependent on water pH
- DOM concentration does not influence the binding

GRAPHICAL ABSTRACT



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ABSTRACT

Information on how key environmental conditions such as natural dissolved organic matter (DOM) and water pH alter the possible risks posed by pharmaceuticals (PPCPs) is still scarce. In our previous study, the presence of natural DOM at high pH reduced the toxicity of a mix of waterborne PPCPs to algae. DOM-complexation and pH effect on speciation of the more hydrophobic and neutral compounds of the mix was suggested to be driving this behaviour. However, the study design did not allow the verification of this hypothesis. Here, the DOM- PPCPs interaction at different pH was investigated for 6 PPCPs through equilibrium dialysis, under the same conditions of DOM and pH as our previous study. Association with DOM was confirmed for the more hydrophobic PPCPs at high pH. The results suggest the binding was driven by i) the presence of carboxylic groups of PPCPs, ii) high pH shifting the structural configuration of DOM, making it more suited to bind some of the PPCPs. A non-linear change of binding capacity with increasing DOM concentration was also observed among the tested PPCPs.

1. Introduction

The widespread occurrence of anthropogenic contaminants in freshwater ecosystems is a major concern (Pal et al., 2010). Emerging contaminants such as the group of pharmaceutical and personal care products (PPCPs (Boxall et al., 2012)), widely used and continuously discharged in treated and untreated wastewater (Schwarzenbach

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et al., 2006), are particularly worrisome as they are biologically active at low concentration. In the environment, PPCPs can interfere with fundamental metabolic pathways (e.g. chlorophyll-a and lipid synthesis) in non-target organisms such as freshwater phytoplankton (Zhang et al., 2012, 2019); this could possibly translate into severe repercussions for the functioning of freshwater ecosystems (Arnold et al., 2013), considering the key ecological functions underpinned by microalgae. Knowledge of freshwater biota sensitivity to these stressors is mostly derived from experiments conducted under controlled laboratory conditions (OECD, 2009). There is a paucity of information on how the risk posed by these contaminants is modulated by the prevailing environmental conditions (Holmstrup et al., 2010; Laskowski et al., 2010). Dissolved organic matter (DOM) is present in all surface freshwaters (Leenheer and Croue', 2003) and has the ability to bind, adsorb and/or transform contaminants by forming complexes that are too large or too polar to cross biological membranes (Lipnick, 1995). Through this process, DOM (generally analyzed as concentration of dissolved organic carbon – DOC) may reduce the bioavailable fraction and the toxicity of contaminants (Chen et al., 2017; Pan et al., 2009; Rowett et al., 2016).

The DOM binding affinity (usually expressed as distribution coefficient K_{DOC} or K_d) can be controlled by several factors, such as the water chemistry (i.e. pH), the physicochemical properties of the compounds (i.e. hydrophobicity, presence of functional groups) (Ashauer and Escher, 2010; Behera et al., 2010; Sun et al., 2020) and of the DOM (i.e. molecular size, aromaticity, presence of functional groups and concentration; Gu et al., 2007; Tanaka et al., 2005). For example, a change in water pH can affect the compound-DOM complexation by altering the compound and/or the DOM molecular configuration (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999). The relationship between DOM binding capacity and its concentration is also unclear. For instance, while linear correlations are generally reported (Burkhard, 2000; Krop et al., 2001), other studies observed that increasing concentrations of DOM could generate tighter molecular rearrangements, preventing the chemical compounds accessing the more hydrophobic areas of the DOM where binding generally takes place (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). Hence, the effect of these complex interactions on toxicity of contaminants cannot be predicted easily. This is particularly important in boreal freshwater ecosystems. Here, climate and land-use changes - together with recovery from past acidification - have caused an increase in the levels of DOM and altered the water pH over recent decades, a process also known as water browning (Monteith et al., 2007; Williamson et al., 2015).

The influence of the interaction between DOM and water pH on the toxicity of PPCPs has been a subject of research (Pan et al., 2009). In a recent study (Rizzuto et al., 2020), we found that the toxicity of an environmentally realistic mix of 12 PPCPs to algae was reduced in water with low levels of natural DOM and high pH. We suggested that high pH conditions increased the DOM-PPCPs binding affinity by controlling the physicochemical properties of both PPCPs and DOM. In addition, a direct effect of DOM in hindering algal growth was observed with a non-linear dependence on DOM concentration (Rizzuto et al., 2020). However, that study design did not allow evaluation of the hypothesis on the mechanisms of interaction between PPCPs and DOM.

In this study we therefore explicitly addressed the interaction between PPCPs and the natural DOM at two different pH. Six PPCPs with demonstrated toxic effects on phytoplankton, with a range of different physicochemical properties were selected from the original mix; the same pH values, natural DOM (extracted from the same lake, Gjessing et al., 1999) and concentrations were used as in the previous study (Rizzuto et al., 2020). An equilibrium dialysis technique was used to investigate the chemical-DOM binding (Akkanen et al., 2001; Akkanen and Kukkonen, 2001). With this method, the contaminant molecules can freely diffuse across the dialysis membrane, whereas the larger-sized DOM complexes are restricted to one side of the membrane. This method has been commonly used, but usually with a commercially available DOM only (i.e. Suwannee River, isolated humic or fulvic acids, Böhm et al., 2016). However, natural DOM is polydisperse, and may contain different constituents with varying levels of hydrophilicity, as well as several functional groups with different chemistry (Leenheer and Croue', 2003). Hence, while it is important to have a standardized DOM and/or to investigate the influence of different functional groups, it is also crucial to test the effects arising from DOM naturally occurring in local freshwater ecosystems that is relevant to the investigation (Akkanen et al., 2001). In addition, one technical hindrance recognized by several authors concerns the pore size of the membrane generally used in these experiments (>1000 Da); this could cause leakage of smaller molecular size DOM across the dialysis membrane, leading to an over-underestimation of the binding effect of DOM (Akkanen and Kukkonen, 2003). In the present study we overcame these limitations by using: i) natural DOM isolated from a boreal catchment (Gjessing et al., 1999), containing virtually the full spectrum of constituents native of boreal systems; ii) hydrophilic semipermeable membranes with a smaller pore size (100–500 Da), enabling only relatively small and free chemicals (e.g. in the range of the PPCPs used here) to permeate.

Results from the equilibrium dialysis studies are used to test the hypotheses arising from our previous study, considering the implications for the potential effects on freshwater ecosystems.

2. Materials and methods

The association of a mix of 6 PPCPs to three levels of natural DOM (0, 5 and 15 mg L^{-1} DOC), at two water pH values (6.5, 8) was tested using the equilibrium dialysis technique (Fig. 1).

2.1. Selection of experimental conditions

2.1.1. Chemical compounds

Six PPCPs were selected from the mix used in our previous study (Rizzuto et al., 2020), namely: Atenolol (ATE), Sulfamethoxazole (SMX), Furosemide (FUR), Clarithromycin (CLA), Bezafibrate (BEZ), and Diclofenac (DCF) (chemical structures in Fig. S1). They are among the most commonly detected PPCPs in European wastewaters and freshwaters (concentrations in natural freshwaters ranging from 0.1 to 380 μg L^{-1}, Table S1). This selection provides: i) a broad range of hydrophobicity (measured as the octanol-water partition coefficient - log K_{ow}); ii) a range of acid dissociation constant (pKa) values; and iii) presence of different functional groups on the analytes (Table 1). Expected concentrations of each chemical at the end of the experiment at equilibrium conditions (C_E, Table 1) are within the range of those detected in European freshwaters (Table S1).

2.1.2. DOM and pH

The DOM used in this experiment was previously isolated through reverse osmosis from the water of the Hellerudmyra tarn (Norway) (Gjessing et al., 1999), and donated by the University of Oslo (Norway). Hellerudmyra tarn is a small catchment (0.08 km^2) that provided DOM samples for the IHSS Nordic Fulvic and Humic Reference Material (Gjessing et al., 1999). The most relevant physicochemical properties of this natural DOM are reported in Table 2. Other properties of the DOM were detailed in Gjessing et al. (1999). The levels of DOM applied here represent low to medium-high concentrations typically found in Northern European lakes (Henriksen et al., 1998). The pH levels were used to represent the range typically found in Northern European Lakes (Henriksen et al., 1998). pH was monitored in all experimental units. No significant change in pH occurred in any of the units during the experiment (data not shown).

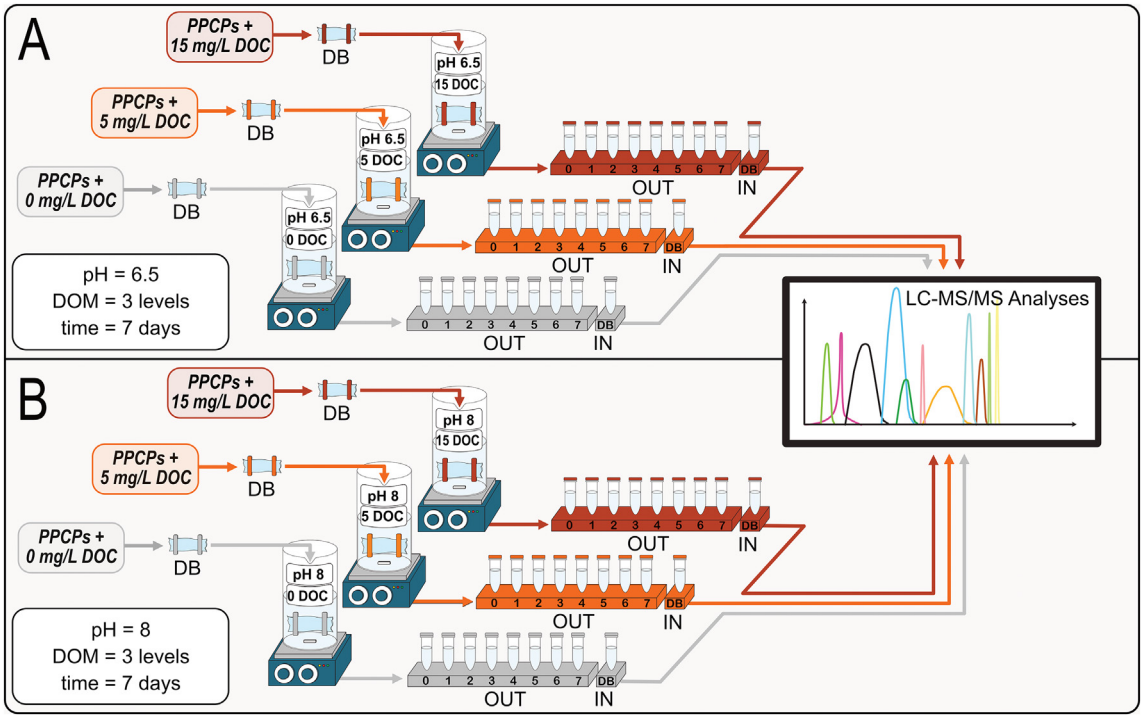

Fig. 1. Experimental design. The association of the mix of 6 PPCPs to DOM (0, 5 and 15 mg L^{-1} DOC) was tested through equilibrium dialysis technique at pH 6.5(A) and 8(B). DB; dialysis bags, OUT; samples collected each day over 7 days outside the dialysis bag, IN; samples collected inside the dialysis bag on the seventh day of the experiment.

2.2. Experimental setting

2.2.1. Preparation of PPCPs and DOM working solutions

Analytical standards of the 6 compounds were purchased from Sigma-Aldrich (USA) and individually diluted in methanol (Sigma-Aldrich) to create 4 stock solutions of 1 mg mL^{-1} for ATE, CLA, BEZ and DCF, and 2 stock solutions of 100 μg mL^{-1} for SMX and FUR. To prepare the working solution of the mix, 100 μL from the ATE, SMX, FUR, CLA and BEZ stock solutions and 200 μL from DCF stock solution was spiked into an amber glass vial, to reach the following concentrations in 10 mL total volume: ATE, CLA and BEZ 10 μg mL^{-1}, DCF 20 μg mL^{-1} and SMX and FUR 1 μg mL^{-1}. The DOM working solution was prepared by weighing 10 mg of dry DOM and adding 10 mL of MQ water to reach a concentration of 1 mg DOM mL^{-1}.

2.2.2. Preparation of artificial water solutions at two different pH

Four litres of soft artificial water (SAF) was prepared by adding MQ water with 1.17 g NaCl to reach 0.01 M (0.58 g L^{-1}) ionic strength, commonly detected in boreal freshwaters. The solution was then split in two 2 L bottles, and then adjusted by titration with HCl or NaOH to reach 6.5 and 8 respectively. The SAF solution was used inside and outside the dialysis bags.

2.2.3. The experimental units

Cellulose ester dialysis bags, with a flat-width of 31 mm and molecular cut-off of 100–500 Da (Spectra/por, Spectrum Europe, Breda, The Netherlands), were cut to 10 cm lengths and thoroughly washed with Milli-Q water. 460 μL aliquots of the PPCPs mix working solution were spiked in each dialysis bag. For the control units with no DOM, SAF

Table 1
Physicochemical properties of the 6 investigated compounds.

PPCPs	Use	Log K_{ow}	Functional groups	Molecular weight (g mol^{-1})	Water solubility (mg L^{-1})	pKa	C_E (μg L^{-1})
Atenolol (ATE)	Beta-blocker	0.16 (a)	Amine	266.34	13,300	9 (f)	22
Sulfamethoxazole (SMX)	Antibiotic	0.89 (a)	Isoxazole	253.28	610	1.6/5.7 (g)	2.2
Furosemide (FUR)	Diuretic	2.03 (b)	Chlorobenzoic	330.74	73.1	3.9 (h)	2.2
Clarithromycin (CLA)	Antibiotic	3.16 (c)	Amine	747.96	1.63	8.99 (c)	22
Bezafibrate (BEZ)	Lipid-lowering	4.25 (d)	Carboxylic	361.82	1.55	3.83 (i)	22
Diclofenac (DCF)	Non-steroidal anti-inflammatory	4.51 (e)	Carboxylic	296.14	2.37	4.15 (b)	44

For reference on Log K_{ow} and pKa values:
a. (Hansch et al., 1995),
b. (Sangster, 1997),
c. (McFarland et al., 1997),
d. (Tang et al., 2014),
e. (Avdeef, 2005),
f. (O'Neil, 2013),
g. (Boreen et al., 2004),
h. (Khan and Ongerth, 2004),
i. (ChemAxon, 2021).

Table 2
Physicochemical properties of the natural DOM used in this study when prepared at typical concentration found in the source lake (see Gjessing et al., 1999 for details).

pH	Conductivity ($mS\ m^{-1}$)	Colour ($mg\ Pt\ L^{-1}$)	UV absorbance 254 nm	$(SUVA_{254})^a$ ($mgC \times 10^2$)	%Corg[b]	molecular weight (Da)	C_{ar}/C_{al}[c]
5.17	2.49	166	0.813	4.59	50.3	3900	0.22

Lake DOC concentration typically of 17.7 $mg\ L^{-1}$.
[a] Specific UV – Absorbance at 254 nm.
[b] Percentage of Carbon content.
[c] Ratio of aromatic to aliphatic carbon.

was then added to each bag, to reach the final volume of 10 mL. For the 5 $mg\ L^{-1}$ and 15 $mg\ L^{-1}$ DOC units, 170 μL and 510 μL of DOM working solution were spiked into the respective bags, before adding SAF to reach the final volume of 10 mL. Dialysis bags were sealed with standard closures (Spectra/por, Spectrum Europe, Breda, The Netherlands) and placed into a 200 mL of SAF solution (Fig. 1). After adding glass-coated metal stirrer bars (20 mm) the experimental units were closed on top with Teflon linen plugs, and placed on a magnetic stirrer (Multistirrer15, Progen Scientific, UK) in the dark at 19 ± 1.2 °C for 1 week. The experimental procedure was repeated at both pH levels (6.5, 8), in triplicates (Fig. 1). Every day for 7 days, 2 mL samples were collected from the units (externally from the dialysis bag) and split into two 1 mL aliquots for the determination of PPCPs and to check for a potential break-through of DOM through the dialysis membrane, respectively. On the last day of the experiment, 1 mL sample was also collected from each unit, from inside the bags. Samples for chemical analyses were filtered through a 0.2 μm syringe, placed into a 2.5 mL amber glass vial, and stored at −20 °C until analyses. Chemical analyses were carried out through direct injection in LC-MS/MS (Shimadzu, 8081) following the method reported in Text S1. MS/MS acquisition parameters are reported in Table S2. The samples for DOM loss were processed immediately following the method reported in Text S2. No detectable release of DOM from the dialysis bags was observed at either pH level (Fig. S2). Mass recovery (Text S3) and mass loss due to adsorption of the PPCPs to all the components of the experimental units (dialysis bag, glassware, clips and stirrer bars; Text S4) was also tested.

2.3. Data management scheme and quality assurance criteria

Data were treated according to the scheme presented in Fig. 2. In summary, quality thresholds for reproducibility, mass balance closure in the experimental units, adsorption of PPCPs on the glassware and membranes and quality criteria to verify transmembrane equilibrium were considered as described in the following sections:

2.3.1. Reproducibility, mass balance and adsorption to glassware and membrane

The quality of replicates, mass recovery of the compounds (i.e. mass balance closure), and their mass loss due to adsorption to components of the experiment unit were checked both inside (internal wall of the bag, hereon refereed as A_{in} (μg)) and outside the bag (sum of external wall of the bag, glassware, clips and stirring bars, A_{out} (μg)). Acceptable mass recovery was set to be at 100 ± 10% of the mass of the compounds. Equilibrium dialysis studies do not generally report adsorption parameters, because they are based on the assumption that any chemical adsorbed to the bag, the glassware or other components should not interfere with the equilibrium between the compound inside and outside the bag (Akkanen et al., 2001; Akkanen and Kukkonen, 2003). However, excessive adsorption could impose a gradient preventing the compound from reaching equilibrium, such that the true effect of DOM may not be observable. In addition, our experimental setup differs from the others since we used a smaller pore size of the dialysis membrane (100–500 Da) to prevent DOM leakage, which can potentially influence the adsorption of the compounds by slowing their diffusion. Hence, mass loss due to adsorption (A_{in}, A_{out}) was included in the QA/QC parameters and set as acceptable in the range of 0–35% of the total added mass of individual PPCPs.

2.3.2. Equilibrium

A condition for the data to be considered for the next steps of the assessment of DOM complexation was that the concentration of PPCP in the control units reached equilibrium across the dialysis membrane by the end of the experiment. The criteria used to establish equilibrium conditions were:

- No significant differences were found between the concentration of the compound inside the control dialysis bag, C_{in} ($\mu g\ L^{-1}$), and concentration outside the control bag, C_{out} ($\mu g\ L^{-1}$). C_{in} could include both complexed and free compound, while C_{out} only represents free compound. Because the environments inside and outside the dialysis bags had very different volumes (10 mL inside vs. 200 mL outside), the mass (μg) of each chemical was calculated inside ($M_{D/in}$) and outside ($M_{D/out}$) the bag to avoid any potential under-overestimation induced by dilution. The mass was calculated from the concentrations

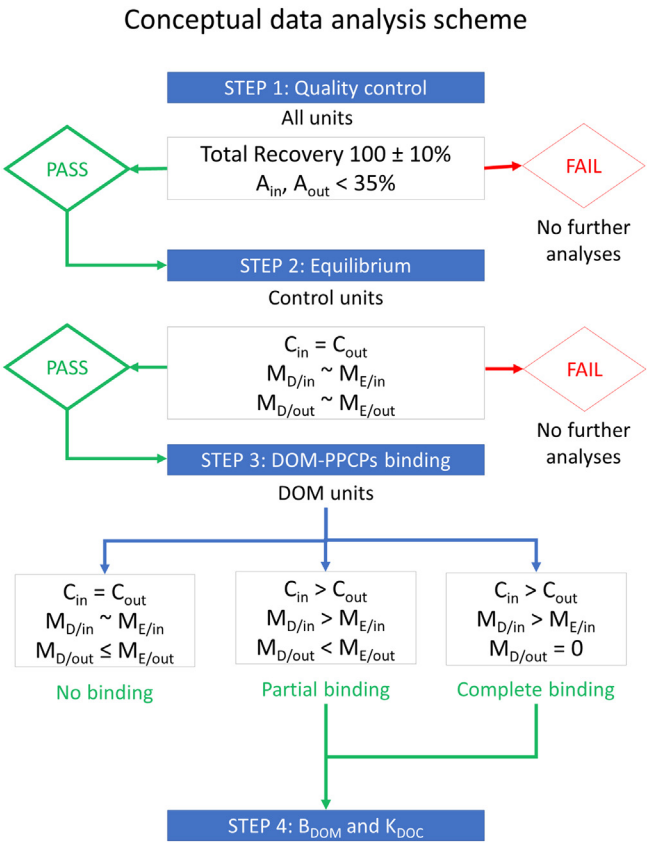

Fig. 2. Conceptual data analysis scheme. C_{in} and C_{out} are the concentrations of each PPCP inside/outside the dialysis bags. $M_{D/in}$ is the mass of each PPCP detected inside the dialysis bag. $M_{D/out}$ is the mass of each PPCP detected outside the dialysis bag. $M_{E/in}$ is the mass of each PPCP expected inside the bag of the control units at equilibrium.

detected inside and outside the bag, not including any associated with the glassware and dialysis bag.
- The value of $M_{D/in}$ and $M_{D/out}$ should not be significantly different from the mass expected inside and outside the dialysis bag of the control units at the end of the experiment ($M_{E/in}$ and $M_{E/out}$, respectively). The values of $M_{E/in}$ and $M_{E/out}$ were calculated from the values of the final concentration expected inside and outside the dialysis bag of the control units at the end of the experiment (C_E), assuming that C_{in} equilibrated with C_{out}, and allowing A_{in} and A_{out} values ranging 0–35%.

Failure to meet these conditions for the control units prompted the exclusion of the compound at that pH level from the study.

2.3.3. DOM-PPCPs binding

When the PPCPs in the control units reached equilibrium and all the quality criteria were fulfilled, the DOM-PPCPs binding was also assessed for the units containing DOM. Theoretically, if DOM bound the compounds, a significantly higher concentration will be detected inside the dialysis bags than outside, because complexation by the DOM prevents the compound crossing the membrane of the dialysis bag due the larger size. Analogously to the comparison of $M_{D/in}$ and $M_{D/out}$ performed on the control units to assess equilibrium conditions, a difference of $M_{D/in}$ and $M_{D/out}$ whereby $M_{D/in}$ being in significant excess of $M_{D/out}$ was deemed as one required piece of evidence for partial or complete binding of PPCPs to DOM. Furthermore, to rule out the confounding factor of different dilution between the bag inner and outer environment (and following the conceptual scheme of Fig. 2), another condition for PPCP binding to DOM was that $M_{D/in}$ should be higher than $M_{E/in}$ for the experimental units with DOM and, concurrently, $M_{D/out}$ should be lower than $M_{E/out}$.

$M_{E/out}$ was calculated on the same assumptions used for $M_{E/in}$ (see paragraph *2.3.2 Equilibrium*). In summary, after checking for equilibration in the control units, three scenarios were considered to determine complexation in the units containing DOM:

- No binding: $C_{in} \sim C_{out}$; $M_{D/in} \sim M_{E/in}$, and $M_{D/out} \leq M_{E/out}$.
- Complete binding: $M_{D/in}$ represented 100% of the PPCP mass spiked in the experimental unit, $M_{D/in} > M_{E/in}$, while $M_{D/out} = 0$.
- Partial binding: $M_{D/in} > M_{E/in}$ and $M_{D/out} < M_{E/out}$.

The magnitude of the effect of DOM was also tested by calculating the conditional distribution coefficient (K_{DOC}, L g^{-1}) at both levels of DOM. K_{DOC} was calculated as follows (from (Buschmann et al., 2006)):

$$K_{DOC} = \frac{[C_{in}] - [C_{out}]}{[C_{out}]\ x\ [DOC]} * 1000$$

where the term $[C_{in}]$-$[C_{out}]$ is the concentration of compound complexed with DOM and $[C_{out}]$ is the concentration of free (unbound) compound. DOC and DOM values can be inter-converted in the equation by using the DOM's % of C content value of 50.3 (see Table 2).

The percentage of bound compound (B_{DOM}), defined by mass of bound compound divided by the total mass of the compound inside the bag, was calculated as follows:

$$B_{DOM} = \frac{M_{in} - C_{out} * V_{in}}{M_{in}} * 100$$

where V_{in} is the solution volume inside the dialysis bag and $C_{out}{}^{*}V_{in}$ is the mass of unbound inside the bag. M_{in} is the total mass of compound inside the bag.

2.4. Statistical analyses

Data analyses and statistics were conducted using R (version 3.5.1) statistical software (R Core Development Team, 2015). The single comparison analyses between C_{in} and C_{out}, K_{DOC}, and B_{DOM} between different DOM levels were tested by one-way ANOVA test. The graphs were prepared using the R package "ggplot2" (Wickham, 2006).

3. Results and discussions

3.1. Step 1: quality control

All the compounds had 100 ± 10% total mass recovery at all pH and DOM levels in all units, apart from 3 outliers (Tables S3-S9). The optimal recovery rates shown by each targeted PPCP (Table S3-S9) indicated that ion suppression, a technical hindrance that can affect the accuracy of LC-MS/MS analyses using direct injection (Antignac et al., 2005; George et al., 2018), was negligible. Results from ATE, SMX, FUR, BEZ and DCF showed that A_{in} and A_{out} were minor or acceptable (2–35% mass loss (Tables S3-S8). In contrast, CLA showed higher A_{in} and A_{out} than the other compounds (Table S6), yielding values of ca. 32% at pH 6.5, and ca. 70% at pH 8 (Table S6). Higher adsorption of this compound at pH 8 probably links to its relative hydrophobicity (log K_{ow} 3.16 in the associated form with a pKa of 8.99). At pH 8, 91% of CLA was expected to be present in the solution in the associated form, compared to 99.7% at pH 6.5. The presence of more neutral (and consequently more hydrophobic) forms of the compound available at pH 8 may have enhanced its adsorption to the glassware, plastic clips or stirrer bars. The chemical configuration of CLA is very complex (see Fig. S1) and offers the presence of different domains that can create H-bonds. At the same time, H-bonds are also sensitive to pH, as a consequence of the pH-induced charge density and the conformation change of CLA or of the dialysis membrane. Variable adsorption of CLA to glassware has been reported previously (Wibawa et al., 2003). Daily monitoring of water pH reported no significant changes (data not shown), which excluded possible confounding effects induced by water pH drifting during the experiment.

3.2. Step 2: equilibrium

Secondly, we checked transmembrane equilibration in the control units, based on the criteria mentioned above (Fig. 2). C_{out} and C_{in} measured on the seventh day are depicted in Fig. 3 for the different DOM and pH conditions (for C_{out} during the seven-day experiment see also Fig. S3). The control units displayed no significant differences between C_{in} and C_{out} on day 7 at pH 6.5 or pH 8 for ATE, SMX, CLA (at pH 6.5 only), BEZ and DCF (Fig. 3, Table S10). Furthermore, in compliance with the set quality assurance criteria, the $M_{D/in}$ values of these compounds observed in the control units were not higher than $M_{E/in}$ (Table S3-S4, S6-S8). This confirms that the equilibrium conditions were met for free chemical compounds (not complexed) at both pH levels. In contrast, significant differences were observed for FUR at pH 8, where C_{in} in the control unit was significantly higher than C_{out} (Fig. 3, Table S10), while no differences were observed at pH 6.5. In addition, $M_{D/in}$ of this compound was approximatively 20% higher than $M_{E/in}$ (Table S5). These results indicate that FUR did not reach equilibrium before the end of the experiment at pH 8. Water pH was found to significantly influence the behaviour of FUR ($F = 5.66$, $p < 0.05$). This is not surprising because FUR is a relatively large molecule, with MW close to the lower boundary of the molecular cut off of the membrane and includes a carboxylic group with an estimated pKa = 4.25. Beyond this ionizable functional group, FUR contains two domains capable of forming N-H···O hydrogen bonds. These domains can obviously form hydrogen bonds with groups of the cellulose ester, making FUR-membrane interactions sensitive to pH. Furthermore, the sulphamoil group at position 2 of the chlorobenzoic acid domain of FUR and the amine group in position 5 interacting with the oxygen of the furan group will both affect steric configuration and intramolecular interaction of this compound, depending on solution pH. Because of these characteristics FUR is an example of a conformational polymorph (Thakuria et al., 2017). Examples of the effect of pH on steric

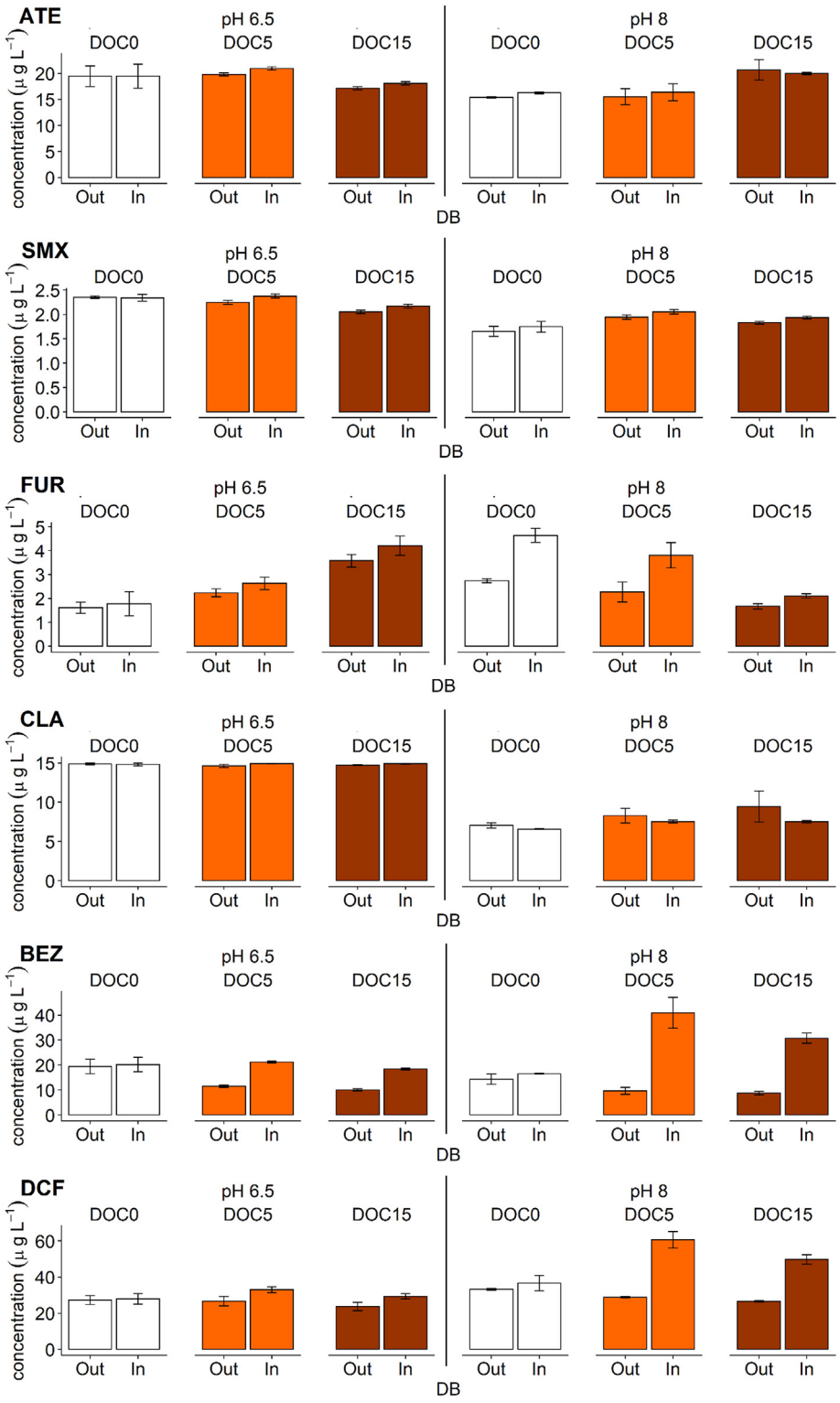

Fig. 3. Concentration outside (C_{out}) and inside (C_{in}) the dialysis bag (DB) on the last day of the experiment for the six investigated PPCPs, at three levels of DOM (0, 5 and 15 mg L^{-1} DOC) and two levels of pH (6.5, 8). Error bars represent standard deviation.

arrangements of compounds used in drugs formulations are illustrated elsewhere (Frenkel et al., 2005). FUR at pH 8 failed to meet the equilibrium conditions and was therefore excluded from further analysis and discussion. The compounds reaching equilibrium (ATE, SMX, CLA at pH 6.5, FUR at pH 6.5, BEZ and DCF) were admitted to the next procedural steps, where the DOM-PPCP binding was assessed.

3.3. Step 3 DOM-PPCPs binding

The third step of the data processing scheme was to assess the degree of binding with DOM for those chemicals that passed steps 1 and 2. ATE, SMX, CLA (pH 6.5) and FUR (pH 6.5) did not show significant differences between C_{in} and C_{out} for different DOM levels (Table S11–12),

which indicates no binding according to the experimental design criteria and conceptual analysis scheme in Fig. 2. In contrast, BEZ and DCF showed significantly higher C_{in} than C_{out} in the water containing DOM (Fig. 3, Table S11–12). $M_{D/in}$ of the DOM units was significantly higher than $M_{E/in}$ for both the compounds (Tables S7-S8). In addition, the $M_{D/out}$ was >0 for both compounds at both DOM levels (Table S7-S8), indicating partial binding with DOM, in line with the conceptual analysis scheme (Fig. 2). Complete binding was not observed for any of the investigated compounds, according to the conceptual analysis scheme.

3.4. Factors influencing DOM-PPCPs binding affinity

3.4.1. Physicochemical properties of PPCPs and the role of DOM composition

Generally, the interaction between organic contaminants and DOM can be driven by different processes which may act both individually or synergistically. These may be hydrophobic interactions, weak H-bond interactions and covalent bonds.

The linear free energy concept, whereby the DOM-contaminant affinity – K_{DOC} or K_d - is positively correlated with the degree of hydrophobicity of contaminants – K_{ow} - (Pan et al., 2009) describes hydrophobic interactions. The results broadly indicate an influence of this interaction, with the higher hydrophobicity compounds (DCF and BEZ, log K_{ow} of 4.5 and 4.25, respectively – Table 1) showing association/partial binding with the DOM. In contrast, no DOM-binding was observed for the 4 compounds with lower hydrophobicity (ATE, SMX, FUR (pH 6.5) and CLA (pH 6.5), log K_{ow} of 0.16, 0.89, 2.03 and 3.16, respectively – Table 1). Nevertheless, interpretation only based on hydrophobic interaction is over simplistic. Firstly, because these compounds all have weak acid groups which can interact with electron donors in the DOM and functional groups (such as amines) that can form N-H···O hydrogen bonds with oxidized groups in the DOM. Furthermore, all these PPCPs have aromatic rings which may engage in ion-dipole induced or dipole-dipole induced interactions with a range of DOM domains (especially aromatic and carboxylic groups as well as ketones, abundant in DOM) (Holbrook et al., 2004). In principal, cation exchange, cation bridging, surface complexation, and hydrogen binding can represent different forms of interaction between these PPCPs and DOM (Hernandez-Ruiz et al., 2012; Kwon and Armbrust, 2008; Pan et al., 2009). This is indicated by the B_{DOM} and K_{DOC} results (Table S13), that show BEZ having a higher degree of association with DOM than DCF, despite the latter having greater hydrophobicity. BEZ showed average B_{DOM} and log K_{DOC} ranging 45–74% and 1.98–2.52 respectively, while DCF indicated average values of 49% for B_{DOM} and 2.05 for log K_{DOC} (Table S13). The association of DCF and BEZ with DOM has been reported previously (Lu et al., 2018). Yamamoto et al. (2003) reported that other moderately lipophilic pharmaceuticals - 17β-estradiol, estriol and 17β ethynylestradiol (log K_{ow} of 3.94, 2.45 and 3.67, respectively) - with carboxyl groups similar to DCF - bound to DOM with sorption energy comparable to that of hydrogen bonding. Other studies report evidence of BEZ interacting with DOM, under experimental conditions designed to study degradation or removal of PPCPs from drinking water (Maeng et al., 2012; Vieno et al., 2010). To the best of our knowledge, the results presented here are the first on the interactions of naturally occurring DOM with BEZ.

Our study differs from others in reporting evidence of weak interactions for the other 4 compounds with DOM. Yamamoto et al. (2005) showed very low sorption of ATE to DOM (0.2% bound fraction, through fluorescence quenching observations) probably due to specific sorption forces other than hydrophobic interactions, or π- π interactions with Gibbs free energy contributions as an alternative (Delgado et al., 2015; Keiluweit and Kleber, 2009; Zeng et al., 2012). Some other evidence suggested that binding mechanisms of sulphonamide antibiotics such as SMX tended to be hydrophobic partitioning, despite their low K_{ow} values (Chen et al., 2017). FUR was found to form complexes with fulvic acids, even though the mechanism behind the complexation remains unclear (Prakash Agarwal et al., 2008). Interactions of CLA with terrestrial humic acids (Elliot soil humic acid) through cationic species – low proton affinity sites of humic acids have also been reported (Christl et al., 2016). Despite their relevance for the topic treated in the present studies, none of these cited works used the equilibrium dialysis membrane method or were directly focused on investigating DOM-PPCPs binding. Rather, they were aimed at investigating the photodegradation of the compounds (Zeng et al., 2012), or chemical removal from drinking waters (Delgado et al., 2015; Zeng et al., 2012). Hence the present study introduces novel elements compared to previous assessments. There is no claim that the present approach and results can shed full mechanistic light on the physicochemical processes controlling the interaction between PPCPs and DOM. The focus here was primarily on assessing the occurrence of binding and the potential relevance for exposure and risk assessment, while providing some fundamental evidence of the role of environmental conditions (e.g. by means of varying pH and DOM levels) and of the complexity of such interactions.

Previous assessments of the PPCPs-DOM interactions utilized commercial DOM standards such as Suwannee River Fulvic Acid, Suwannee River Humic Acid, Nordic Lake Fulvic Acid and Nordic Lake Humic Acid (Chen et al., 2004; Delgado et al., 2015; Keiluweit and Kleber, 2009; Zeng et al., 2012; Zhu and Pignatello, 2005). In the present study natural DOM extracted from a boreal lake was utilized. Chemical composition and the molecular configuration of DOM are key to determine modalities and level of interaction with other chemical species in the solution (Chin et al., 1997; Tanaka et al., 2005). The binding affinity of DOM was previously shown to positively correlate with its aromaticity and/or molecular weight (Akkanen et al., 2004; Ripszam et al., 2015; Tanaka et al., 2005). The natural DOM used in this experiment has a medium-high degree of aromaticity ($SUVA_{254}$ 4.59) and molecular weight (3900 Da) compared to that used in previous studies (Akkanen et al., 2004; Ripszam et al., 2015). Relatively high molecular weight DOM in humic-rich lakes was also reported in other studies (Tulonen et al., 1992; Wang et al., 2020).

3.5. Effects of pH on DOM-PPCPs binding affinity

The results indicated that the association of DOM with BEZ and DCF was influenced by the water pH. BEZ had higher C_{in} than C_{out} at both levels of DOM, at pH 6.5 (ANOVA, $F = 227.9$, $p < 0.001$, Table S11, S12) and 8 (ANOVA, $F = 101.8$, $p < 0.001$, Table S11, S12). However, the results showed that the water pH affected the binding of the DOM and BEZ differently. While at pH 6.5 the $M_{D/out}$ was lower than $M_{E/out}$ (which is in line with our experimental criteria), the $M_{D/in}$ value was lower than the $M_{E/in}$ (Table S9, S7). This could be caused by the higher adsorption to the dialysis bag observed at lower pH, as indicated by the higher A_{in} at pH 6.5 compared to pH 8 (Table S9, S7). In contrast, at pH 8 the $M_{D/in}$ yielded significantly higher values than $M_{E/in}$ at both DOM levels, and the $M_{D/out}$ was lower than $M_{E/out}$ (Table S9, S7). BEZ yielded significantly lower B_{DOM} and log K_{DOC} values at pH 6.5 (45% and 1.98, respectively; average values between the two DOM levels) compared to pH 8 (74% and 2.52 respectively; average values between the two DOM levels) (Table S13). Hence, according to the BEZ results at pH 6.5 and 8, and criteria for binding (Fig. 2), BEZ associated with DOM at both pH levels, but the interaction was stronger at pH 8.

DCF showed significantly higher C_{in} compared to C_{out} at pH 8 ($F = 82.53$, $p < 0.001$), while the difference between C_{in} and C_{out} was too small to be significant at pH 6.5 (Table S11). The primary criterion for DOM-association was not met at pH 6.5, which means that no binding occurred. Significant binding with DOM at pH 8 was instead confirmed by $M_{D/in}$ yielding higher values than $M_{E/in}$ at both DOM levels, and by the $M_{D/out}$ lower than $M_{E/out}$ (Table S9, S8). Losses (ca. 35%) were observed from the solution outside the bag due to adsorption of the compound onto to the beaker for both BEZ and DCF (Tables S7-S8).

The effect of high pH increasing the binding with DOM observed for both compounds could be driven by two different processes. The first is a direct effect of water pH altering the ionic configuration and speciation of chemicals (Ashauer and Escher, 2010), changing their chemical properties and/or association with DOM. At higher pH weak acid groups in both PPCPs and DOM will be more in the dissociated form. While this may reduce the contribution of hydrophobic interaction on the binding process, it may simultaneously promote ionic or dipole-dipole interactions. This can explain the results for BEZ. However, BEZ and DCF are weak carboxylic acids (pKa = 3.83 and 4.15, respectively) and the difference in their speciation between pH 6.5 and 8 is negligible. For instance, both BEZ and DCF show 99% of ionized form at both pH 6.5 and 8 (data not shown). Hence, a more likely driving process will be the effect of pH on DOM physicochemical properties and molecular configuration (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999). For example, a change in pH may modulate the speciation of DOM functional groups (Tanaka et al., 2005), altering the fraction of protonated carboxylic groups, modulating the intra- and intermolecular H-bonding and leading to a different binding affinity (Gu et al., 2007; Pace et al., 2012). More acidic environments generally induce a more tightly condensed structure of DOM polymers and colloids, while a more alkaline environment usually causes an expansion of these structures (Pace et al., 2012). It was suggested that the primary mechanism responsible of this shift may not be the change in pH as in H^+ concentration, but a modulation in base cation concentration promoting the expansion and stabilisation of DOM structures (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980; Myeni et al., 1999).

3.6. Effects of DOM concentrations on B_{DOM} and K_{DOC}

The binding of BEZ and DCF with the DOM was not substantially different between the two different concentrations of DOM. The results of the percentage of bound compound, B_{DOM}, showed that the effect of increasing DOM concentration on them was negligible. For instance, B_{DOM} values yielded 76% at the lower level of DOM, and 72% at the higher level for BEZ. For DCF, B_{DOM} was 46% at 5 mg L^{-1} DOC, and 52% at 15 mg L^{-1} DOC (Table S13). These results indicate that the maximum capacity to bind PPCPs by the natural DOM was already reached at 5 mg L^{-1} DOC. The results of the conditional distribution coefficient, $K_{DOC,}$ showed higher values at lower DOM concentrations. For instance, BEZ log K_{DOC} was 2.22 at 5 mg L^{-1} DOC and 1.74 at 15 mg L^{-1} DOC at pH 6.5, and 2.81 at 5 mg L^{-1} DOC and 2.22 at 15 mg L^{-1} DOC at pH 8, while for DCF log K_{DOC} was 2.34 at the lower level of DOM, and 1.76 at the higher one, at pH 8 (Table S13). Generally, log K_{DOC} results should not be affected by the DOM concentration, unless the binding capacity has reached its limit with excessive amount of DOM. The fact that higher amounts of DOM in the solution did not bind more PPCP might be because higher cross interaction of DOM constituents at higher DOM concentrations (Carter and Suffet, 1982) can cause changes in the DOM macromolecular structure (Engebretson and von Wandruszka, 1994; Ghosh and Schnitzer, 1980), hindering contaminant access to binding domains of the DOM. A non-linear binding pattern of DOM has been reported previously, showing decreasing K_{DOC} with increasing concentrations of humic acids (Carter and Suffet, 1982). These results were in line with earlier results reporting lower distribution coefficients with increasing DOM concentrations (Akkanen and Kukkonen, 2003).

The K_{DOC} values for PPCPs vary greatly in literature, depending on the different composition and source of OM and the different methodologies used. Our results were in the range of previously reported studies (Carballa et al., 2008; Lobo et al., 2014; Maeng et al., 2012). Nevertheless, it was not possible to directly compare our results with others because, to the best of our knowledge, no other paper reported K_{DOC} values for BEZ and DCF in water. Many K_{DOC} values have been obtained for the association of BEZ and DCF to DOM in soil, using different methodologies. For example, Lyman et al. (1990) reported a K_{DOC} for BEZ of 2.62 through estimation from K_{OW} values. Barron et al. (2009) reported DCF K_{OC} values of 2.39 in agricultural soil by using combined pressurised liquid extraction and solid phase extraction methods prior to LC-MS/MS. These values are similar to those reported in this paper and also indicate that BEZ may have higher affinity for DOM compared to DCF. Rewitt et al. (2015) reported values of K_{DOC} for BEZ and DCF from batch adsorption experiments with DOM extracted from two different soils, where both compounds had higher K_{DOC} values in the soil with higher pH (4.22 ± 0.01; 7.22 ± 0.05) and higher carbon content (83 ± 0.3; 36.5 ± 2.7 g kg-1). DCF also indicated higher K_{OC} values than BEZ (BEZ; K_{OC} 1.6–2.80, DCF; K_{OC} 2.11–3.33). This is further evidence that organic matter originating from different sources can have different level of interaction with chemical pollutants. The use of different methodologies presented in the above-mentioned studies such as fluorescence quenching, equilibrium dialysis, or batch adsorption can also yield different results.

3.7. Implications for PPCP toxicity assessments

Toxicity tests for informing risk assessments of pollutants are conducted under standardized conditions, which typically do not include an analysis of key environmental variables such as the level of DOM in the solution. Hence, more detailed ecological risk assessments for waterborne PPCPs will benefit from a better understanding of how interaction with DOM under a range of environmentally relevant pH levels influences availability of these compounds. In a previous study (Rizzuto et al., 2020), we found that the inhibition effect of a mix of PPCPs on the growth of a micro-algae population was reduced by the presence of natural DOM at pH 8. We hypothesized that decreased toxicity could be attributed to the formation of less bioavailable/toxic DOM-complexes. As several of the PPCPs in the tested mix were weak acids with pKa ranging from 7.99 to 13, we postulated that pH could have a significant influence in determining the interaction and lead to the reduced toxic effects. The study also showed that the effect of DOM in hindering PPCP toxicity was not linearly dependent on its concentration.

Here, by using the same DOM and pH conditions, we empirically demonstrated that the combined effect of low concentrations of natural DOM (5 mg L^{-1} DOC) and high water pH (8) can control availability of some compounds, especially those that are more hydrophobic and more likely to diffuse through cell walls and biological membranes of algae - such as DCF and BEZ (Del Vento and Dachs, 2009). The present results also help explain the observed toxicological outcomes, in that they show a complexing ability of DOM that is not dependent on DOM concentration and is dependent on pH. Since the binding of DOM with PPCPs results in the formation of complexes which are too large or too polar to cross cell membranes (Chalew and Halden, 2009; Rowett et al., 2016), such interactions can reduce the bioavailability and toxic effect of PPCP mixtures (Alsop and Wilson, 2019; Maeng et al., 2012; Rizzuto et al., 2020), especially for compounds such as DCF, which has a demonstrated toxic effect on algae (Doležalová Weissmannová et al., 2018).

3.8. Conclusions

In summary, the present study empirically confirms the hypothesis emerging from our previous study on the complexing role of DOM for PPCPs, even at low DOM concentrations and at pH typical of freshwater environments during the development of algal blooms (Doležalová Weissmannová et al., 2018; Isidori et al., 2007). These results highlight the importance of considering more realistic environmental conditions when addressing the toxicological effects of PPCPs micro-pollutants, by showing that despite their relatively hydrophilic character, they can establish complex interactions with DOM that occurs in all-natural waterbodies. We believe there is a need to expand knowledge on

micro-pollutants effects on biota in waters, through a better understanding of the influence of the key environmental variables.

CRediT authorship contribution statement

SR, LN, EL, DLB, KCJ and HZ conceived the idea. SR, LN, KCJ and HZ designed the experiment. SR collected and analyzed the data. SR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses and manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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