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Cephalexin-dissolved organic matter (DOM) interaction is believed to hinder cephalexin removal from wastewater, potentially enhancing environmental exposure and antibiotic resistance risk. We demonstrate cephalexin-DOM binding occurs under environmentally-relevant conditions, probe its mechanism and show its impact by reducing adsorption by biochar and clay. This work informs a key process in antibiotic environmental fate and selection of appropriate wastewater treatment adsorbents.

Cephalexin interaction with biosolids-derived dissolved organic matter: binding mechanism and implications for adsorptive removal

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Abstract

Trace levels of the β-lactam antibiotic cephalexin are known to persist through wastewater treatment processes, representing a possible route for promoting antibiotic resistance in environments that receive treated wastewater. Processes that impact antibiotic treatment in wastewater, such as binding to dissolved organic matter (DOM), regulate the environmental fate of antibiotics. Here we studied cephalexin binding onto biosolids-derived DOM using fluorescence quenching. Interaction was studied under varying solution pH (4.0 to 8.0), ionic strength (deionized water and I = 0.1 M) and background cations (Na^+ and Ca^{2+}) to determine binding mechanism(s). We observed no binding at pH = 8.0, while cephalexin binding to protein-like fluorophores in DOM was increasingly strong with decreasing pH from 7 to 4. Conditional equilibrium constants varied considerably with pH, with log *Kc* = 8.48, 7.15 and 5.33 for pH = 4.0, 5.0 and 7.0, respectively. Cephalexin-DOM interaction was insensitive to changes in solution ionic strength and cation charge. These results suggest π-π bonding likely drives cephalexin-DOM binding, with the aromatic group of cephalexin interacting with substituted aromatic groups on DOM. Lastly, we showed reduced adsorption of cephalexin from synthetic wastewater onto biochar and clay by DOM under conditions where cephalexin-DOM interaction occurs. This work demonstrates that cephalexin-DOM interaction likely occurs in aquatic environments, with interaction most strongly controlled by pH. We also show that this binding may influence adsorptive treatment and fate in wastewater streams and, therefore, cephalexin fate in the environment.

Introduction

Cephalexin is a β-lactam antibiotic within the cephalosporin class widely used to treat bacterial infections in human and veterinary medicine. Cephalexin is one of the most commonly prescribed antibiotics in several nations worldwide.[1,2] It is estimated that up to 90% of administered cephalexin is excreted unmetabolized through urine, by which it enters wastewater streams.[3] Given the prevalence of cephalexin use, it has been found in wastewater treatment plant influent globally, with concentrations of 29, 64 and 425 μg L^{-1} reported in Hong Kong, Australia, and Iran, respectively.[4–6] Like many antibiotics, cephalexin removal is incomplete and trace levels of cephalexin may persist in treated wastewater and enter the environment through discharge of wastewater into waterways or land application of treated wastewater.[4,7] As a result of this prevalence, cephalexin fate in wastewater streams is particularly relevant to environmental health, as it may influence the development of antimicrobial resistance through selection pressure in wastewater streams and systems that receive treated wastewater.[8]

Antibiotic fate in soil and water systems may be altered by interaction with dissolved organic matter (DOM) ubiquitous in wastewater streams with concentrations previously reported from 8.3-54 mg C L^{-1} in wastewater plant influent and 2.3-33.6 mg C L^{-1} in effluent.[9,10] Interaction between antibiotics and polyfunctional DOM may occur through several possible mechanisms including, H-bonding, hydrophobic interactions, electrostatic forces, π-π interactions and DOM-antibiotic cation bridging.[11] Formed antibiotic-DOM complexes may have different mobilities, degradability, adsorption behavior and bioavailability than respective antibiotics alone.[12–15] These characteristics are critically important for practical management of antibiotic contamination in wastewater through treatment processes, such as adsorptive removal. For example, it was recently demonstrated that addition of soluble natural organic matter to

water reduced cephalexin, sulfadiazine and amoxicillin retention by a layered, mixed environmental media based (e.g., soil, sand, biochar) wastewater treatment system.[16] Another study showed that the addition of DOM to solution reduced adsorption of the β-lactam antibiotic ceftriaxone from solution by montmorillonite clay.[17] Authors from both studies suggested antibiotic-DOM interactions were fundamental to observed behavior. Antibiotic-DOM interactions and their relationship to antibiotic removal from solution, therefore, are key to understanding antibiotic behavior in DOM-rich wastewater streams.

Despite the widespread prevalence of cephalexin in wastewater and the impact of antibiotic-DOM interaction on antibiotic fate in the environment, the interaction between cephalexin with DOM is unclear. Given the range of functional groups present in cephalexin (e.g., carboxylic, aromatic, amine) and DOM, cephalexin has the potential to interact with DOM through several possible mechanisms, yet this interaction has not been established. The subsequent link of this process to adsorptive removal from solution remains unexplored despite cephalexin-DOM interaction being linked to reducing adsorption by various media. The objective of this study was to address these voids by investigating interaction between cephalexin and an activated biosolids derived DOM. Interaction was studied under a range of solution conditions that may impact binding dynamics and, therefore, be used to better understand underlying reaction mechanisms. This includes studying binding under different pH values in a synthetic wastewater matrix as well as varying ionic strengths and background cation species. Cephalexin-DOM binding was probed through fluorescence excitation emission matrices (EEMs) which allow for monitoring quenching of specific DOM structural components by interaction with cephalexin. Furthermore, this study aimed to probe the influence of DOM in solution on cephalexin removal behavior by clay and biochar, materials with demonstrated

potential for antibiotic treatment in wastewater streams.[18,19] This was assessed through batch adsorption experiments to determine distribution coefficients and removal efficiencies for μg L^{-1} levels of cephalexin.

Materials and Methods

DOM isolation

DOM was extracted from activated biosolids (Milorganite, Milwaukee, WI) by a previously established method (Li et al. 2005). Briefly, DOM was extracted from Milorganite at a 1:10 m:v ratio in deionized ultrapure water (18.2 MΩ cm) for 30 minutes. Suspensions were then centrifuged at 1500 relative centrifugal force for 30 minutes, with the supernatant filtered through a 0.45 μm filter. Filtered DOM was then lyophilized and stored in a desiccator until further use.

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) measurements

Solution attenuated total reflectance Fourier transform infrared (ATR-FTIR) measurements were conducted on a Bruker Invenio-R spectrometer (Bruker Optics Inc., Billerica, MA, USA). Spectra were collected from 4000-400 cm^{-1} at a spectral resolution of 4 cm^{-1} and represent an average of 128 scans. After collection, spectra received an atmospheric compensation, smoothing, truncation from 1800-1000 cm^{-1} and baseline correction. All processing was performed in OPUS 8.5 software (Bruker Optics Inc., Billerica, MA).

DOM-cephalexin binding studies

DOM and cephalexin (Sigma-Aldrich, St. Louis, MO, USA) solutions were made in a synthetic wastewater matrix based on a prior survey of chemistries of wastewaters collected in

Southern California (Goldberg 2017). The synthetic wastewater matrix was composed of 0.19 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.21 g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.088 g L^{-1} $NaHCO_3$, 0.516 g L^{-1} Na_2SO_4, 0.008 g L^{-1} $NaNO_3$ and 0.048 g L^{-1} K_2SO_4. For binding studies, cephalexin solutions were added to DOM solutions to yield DOM-cephalexin mixtures with a fixed DOM concentration of 2.5 mg C L^{-1} and $7.2 \cdot 10^{-5}$ to 5.76×10^{-4} M cephalexin. Although most DOM-organic solute interaction studies implementing fluorescence analysis assume rapid (<5 min) equilibration.[20–22] mixtures were incubated at room temperature for 2 h to ensure equilibrium before fluorescence analyses. The DOM concentration was selected to minimize inner-filter effects and, while these cephalexin concentrations exceed those found in wastewater streams, these concentrations were selected to ensure detection of DOM structures which interact with cephalexin. Studies were conducted at a range of pH values (pH = 4.0, 5.0, 7.0 and 8.0) in synthetic wastewater to probe the influence of DOM and cephalexin protonation on binding behavior across a range of DOM and cephalexin protonation states. The influence of ionic strength and background cation charge on cephalexin-DOM binding was studied in background solutions of ultrapure deionized water (DDIW), I=0.1 M NaCl and I=0.1 M $CaCl_2$. The objective of varying ionic strength was not to match conditions found in wastewater, as binding studies in the synthetic wastewater matrix already reflect this, but to perturb solution conditions and allow for inference of interaction mechanisms.

Fluorescence measurements

Fluorescence excitation emission matrices (EEMs) of DOM, cephalexin and DOM-cephalexin mixtures were collected on a Shimadzu RF-6000 spectrofluorometer (Shimadzu Corp., Kyoto, Japan). Excitation and emission wavelengths ranged from 250-500 and 280-600 nm, respectively, in 5 nm steps. Slit widths were set to 5 nm and scans were collected at a rate of 12000 nm/min. EEMs received inner-filter correction, water blank subtraction, Raman

normalization as well as removal and interpolation of scattering peaks prior to peak-picking analysis. Inner-filter corrections were based on UV/Vis spectra of samples collected from 200-600 nm at 2 nm steps on a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). All processing and analysis of EEMs was performed using the staRdom package in R (Pucher et al. 2019).

Quantitative description of cephalexin-DOM interactions

Fluorescence quenching data were quantitatively described using the Ryan-Weber model:

$$\frac{I}{I_0} = 1 + \left(\frac{I_{QL}}{I_0} - 1\right)\frac{1}{2K_cL_t}\left(1 + K_cL_t + K_c[Q]_t - \sqrt{(1 + K_cL_t + K_c[Q]_t)^2 - 4K_c^2L_t[Q]_t}\right)$$

Where I is the fluorophore fluorescence intensity at quencher total concentration of $[Q]_t$, I_0 is the fluorophore fluorescence in the absence of a quencher, I_{QL} is the fluorescence intensity of the fluorophore-quencher complex, K_c is the conditional equilibrium coefficient and L_t is a binding capacity parameter related to the concentration at which no more quenching will occur.[23,24] All fitting was conducted in Microsoft Excel using a nonlinear least squares fitting approach. While this model was originally applied to metal complexation with natural organic matter species assuming a 1:1 ligand:binding site stoichiometry, this model has notably been used to empirically describe binding processes between natural organic matter and organic contaminants as well.[25,26]

Batch cephalexin adsorption studies

Adsorption studies were conducted to determine the impact of DOM on removal efficiencies and distribution coefficients for cephalexin removal from solution by biochar and montmorillonite. Biochar used in adsorption experiments was produced from air dried date palm

158 leaf petiole collected near Thermal, California. Petioles were cut into small pieces (≈1 cm cubes)
159 prior to pyrolysis. The feedstock was pyrolyzed in a Carbolite Gero TF1–1200 furnace (Carbolite
160 Gero, Hope, UK). Samples were heated under N_2 flow (1 L min^{-1}) from ambient temperature to
161 550 °C at a rate of 5 °C min^{-1}. The sample was pyrolyzed at 550°C for 3 h and cooled to room
162 temperature. After pyrolysis, biochar was hand ground with a mortar and pestle and sieved to 80
163 mesh (180 μm). Biochar was then adjusted to pH = 7.0 using a previously described method to
164 ensure pH drift did not occur during adsorption studies and oven dried at 105°C overnight.[27]
165 Biochar was subsequently characterized by previously described methods.[28] Montmorillonite
166 (SWy-1 Na-montmorillonite, Crook County, WY, USA) used in adsorption experiments was
167 acquired from the Source Clay Minerals Repository (Clay Minerals Society, Columbia, MO,
168 USA). Prior to use in adsorption studies, montmorillonite was subjected to removal of
169 carbonates, free oxides and organic matter, followed by Na-saturation and isolation of <2μm size
170 fraction.[29] Montmorillonite was then lyophilized and stored in a desiccator until use. Biochar and
171 montmorillonite physicochemical characteristics may be found in the supporting information
172 (**Table S1**).

173 For adsorption experiments, 20 mL of synthetic wastewater at pH 7.0 with or without 15
174 mg L^{-1} C Milorganite DOM added and with 100 μg L^{-1} cephalexin was initially added to PTFE
175 centrifuge tubes. 40 mg of either montmorillonite or date palm petiole biochar was then added to
176 tubes to yield a 2 g L^{-1} adsorbent dose. Tubes without added adsorbent were also prepared to
177 assess cephalexin losses to degradation and/or adsorption to centrifuge tubes. No detectable
178 degradation or adsorption was observed under studied conditions. All treatments were conducted
179 in triplicates (n=3). Centrifuge tubes were then shaken at room temperature for 24 h in the dark
180 on an orbital table shaker at 125 RPM. After shaking, tubes were centrifuged at 3620 RCF for 20

minutes and the supernatants were filtered through 0.2 μm PTFE syringe filter before subsequent liquid chromatography-tandem mass spectrometric analysis (LC-MS/MS). For partitioning coefficient studies, distribution coefficients (K_d) were determined by:

$$K_d = \frac{c_s}{c_{aq}}$$

Where K_d is the partitioning coefficient (L kg^{-1}), c_s is the antibiotic concentration in solid phase (μg kg^{-1}) and c_{aq} is the aqueous phase concentration (μg L^{-1}).[30]

Removal efficiencies were calculated by:

$$Removal\ efficiency = \left(\frac{c_0 - c_e}{c_0}\right) * 100\ \%$$

Where c_0 is the initial concentration of antibiotics in solution (μg L^{-1}) and c_e is the concentration in solution after adsorption equilibrium (μg L^{-1}).

LC–MS/MS analysis

LC–MS/MS analyses were performed Agilent 1290 Infinity II HPLC coupled with an Agilent 6470B LC/TQ triple quadrupole MS/MS detector and an AJS electrospray ionization (ESI) interface (Agilent Technologies, Inc., Santa Clara, CA, USA). Waters Atlantis T3 (Waters Corp., Milford, MA, USA), 3μm C18 columns were used (guard column: 2.1 mm × 5 mm; analytical column: 2.1 mm × 50 mm) at a flow rate of 0.3 mL min^{-1}. The column temperature was set to 45°C with 0.5 μL injection volume used. Formic acid (0.1%) was used as mobile phase A and acetonitrile as mobile phase B, with gradient conditions set as follows: 0–1min keep at 95% A, 5% B; 10 min reach at 5% A, 95% B; 15 min reach at 5% A, 95% B; 15–20 min keep at 95% A, 5% B. All analytes were detected in positive ion mode. Lab generated nitrogen was

used as the drying and nebulizing gas, with high purity nitrogen gas (99.999% purity) used as the collision gas. Mass spectrometer conditions were as follows: gas temperature, 325°C; dry gas flow, 10 L min^{-1}; nebulizer, 20 psi; capillary voltage, 4000 V; sheath gas temperature, 400°C; sheath gas flow 11 L min^{-1}. Under these conditions, the retention time of cefalexin was 5.055 min. Collection and treatment of data was performed using Mass Hunter software (Version 10.0). Internal standard (Cefalexin-d5) was added to samples and calibration standards prior to analysis to account for matrix effects.

Results and Discussion

DOM characteristics

The fluorescence landscape of extracted DOM is dominated by a peak at ex 275/em 315 nm previously linked to fluorescence by non-conjugated aromatic structures, such as simple phenolics, nucleic acids and aromatic residues in proteins (i.e., tryptophan and tyrosine) (**Figure 1**).[31,32] The lack of strong features elsewhere in the fluorescence landscape by humic or fulvic-like fluorescence corresponds with a previous study which characterized DOM derived from Milorganite by fluorescence EEMs.[33] These characteristics, along with basic optical and chemical properties (**Table S2**), align well with DOM samples in wastewater previously characterized by fluorescence. These studies highlighted the contributions of soluble microbial products and protein-like constituents to wastewater DOM pools through input and microbial processing of sludge biomass.[34–36]

ATR-FTIR spectra of DOM solutions in synthetic wastewater had features related to several functional groups (**Figure 2**). This included amide/ketone C=O stretching (≈ 1650 cm^{-1}), aromatic C=C/ asymmetric COO^- stretching (≈ 1585 cm^{-1}), amide N-H bending (≈ 1537 cm^{-1}),

symmetric COO^- stretching (≈ 1405 cm^{-1}), COOH/phenol C-O stretching (≈ 1220 cm^{-1}) and C-C/C-N/C-O stretching (≈ 1080 cm^{-1}).[37–39] Relative DOM speciation changed across the pH range used in binding experiments. Namely, the asymmetric and symmetric COO^- stretching peaks near 1595 cm^{-1} and 1400 cm^{-1}, respectively, increased in relative intensity with increasing solution pH. The COOH/phenol feature around 1220 cm^{-1} became relatively lower in intensity with increasing pH, particularly above pH = 5.0. These trends were due to deprotonation of DOM carboxylic groups with increasing pH from 4 to 8, suggesting that proton exchange with carboxylic groups was the dominant process controlling DOM speciation across the pH range utilized in this study. Deprotonation of DOM carboxylate groups with increasing pH results in an increasing net negative charge on DOM with increasing solution pH.[40] DOM protonation state also has implications for the types of interactions DOM may engage in with solutes, including H-bonding, $\pi - \pi$ interactions, electrostatic interactions and ternary complexation.[40,41]

DOM fluorescence quenching by cephalexin

Under all studied conditions except for synthetic wastewater at pH = 8.0, addition of cephalexin to DOM samples led to quenching of the dominant ex 275/em 315 nm peak of DOM solutions (**Figure 3**). Appearance of features from cephalexin (ex 275/em 375 nm, ex 325/em 375 nm and ex 335/ em 425 nm) was also observed at all pH values (**Figure S1**). Quenching of the ex 275/em 315 nm peak indicated that cephalexin interacted with the dominant protein-like constituents within DOM. This is in agreement with previous studies probing the interaction of DOM with other antibiotics, such as carbamazepine,[26] tetracycline,[42] and trimethoprim,[22] which also identified microbial product and protein-like DOM constituents as active binding sites for antibiotics through fluorescence quenching. While the specific quenching mechanism (i.e., a static or dynamic quenching process) was not evident from data collected here, as it would

require additional fluorescence lifetime and/or temperature dependent studies, quenching generally indicates direct involvement of the fluorescent functional groups in the binding process.[43,44] For the case of protein-like fluorescence, this would include simple aromatic, phenolic and heterocyclic DOM functional groups. These groups, along with non-fluorescent DOM groups (e.g., carboxylate, hydroxyl) may interact with antibiotics through several possible mechanisms.[11] To discern which mechanisms may have played a role in cephalexin binding to DOM, studies varying solution conditions were conducted.

pH influence on cephalexin-DOM interaction in synthetic wastewater

DOM fluorescence quenching results in a synthetic wastewater matrix across the studied values of pH = 4.0, 5.0, 7.0 and 8.0 are shown in **Figures 4** and **S2**. Across this pH range, DOM speciation was controlled by the exchange of protons by carboxyl groups, with DOM carboxyl groups becoming increasingly deprotonated with increasing pH across this range (**Figure 2**). Cephalexin has two functional groups that exchange protons: a carboxyl group (pK_{a1} = 2.34) and an amine group (pK_{a2} = 7.08) (**Figure S3**). This indicates that cephalexin was predominantly zwitterionic with a net neutral charge at pH = 4.0 and 5.0 and approximately equivalent proportion of zwitterionic and negatively charged species at pH = 7.0 and 8.0. Fluorescence quenching was observed from pH = 4.0 to 7.0, whereas no quenching was apparent at pH = 8.0. Under conditions where quenching was observed, binding was well described by the Ryan-Weber model (**Figure 4, Table S3**). Conditional equilibrium coefficients increased with decreasing pH, with pH = 4.0, 5.0 and 7.0 having log K_c values of 8.48, 7.16 and 5.33, respectively. Log K_c values showed a clear inverse correlation with solution pH (**Figure S4**) and highlighted an increasingly strong association between cephalexin and DOM with decreasing pH from 7.0 to 4.0. L_t values varied as well across different studied pH values, with $8.57 \cdot 10^{-4}$, 2.48

269 •10^{-3} and 3.99 • 10^{-3} M, observed for pH = 4.0, 5.0 and 7.0, respectively (**Table S2**). The trend in
270 L_t values indicated that the concentration at which binding saturation occurs decreased with pH,
271 aligning with the higher affinity of cephalexin-DOM interaction at lower pH values. The lack of
272 binding at pH = 8.0 was likely from electrostatic repulsion due to the predominant negatively
273 charged cephalexin (**Figure S3**) and the extent of deprotonation of carboxylate functional groups
274 on DOM at pH = 8.0 (**Figure 2**). Both conditions would favor a greater degree of electrostatic
275 repulsion between DOM and cephalexin at pH = 8.0 relative to the lower pH values studied,
276 likely precluding interaction.

277 These observations could potentially be explained by several factors related to the
278 cephalexin-DOM interaction mechanism. Previous reports of binding between other
279 pharmaceuticals and natural organic matter species have also shown this behavior, providing
280 some insight into the possible mechanism. For example, Guo et al. noted greater binding
281 between tylosin and sulfamethazine with humic acid as solution pH decreased from pH 8.0 to
282 3.0.[45] This was attributed to more favorable electrostatic and hydrophobic interactions caused by
283 the antibiotics becoming increasingly positive and charge neutral with decreasing pH,
284 respectively. This would correspond with the speciation of cephalexin, which ranged from
285 majority neutral with small contributions from net positively charged species at pH = 4.0 to
286 neutral at pH = 5.0 and approximately equivalent proportion of neutral and negatively charged
287 species at pH = 7.0 (**Figure S3**). Furthermore, the greater extent of DOM protonation at lower
288 pH values, as observed here (See **Figure 2**), would reduce intramolecular repulsion by
289 negatively charged functional groups (primarily carboxylate), favoring DOM molecules to
290 assume a more condensed and less hydrated molecular conformation.[21,40] Another study showed
291 greater extent of sulfonamide binding to natural organic matter with lower pH.[46] The authors

attributed this to H-bonding of increasingly protonated sulfonamides and natural organic matter with decreasing pH as well as increasingly favorable π-π interactions, which become more favorable as aromatic substituent groups become increasingly protonated. Increasing DOM functional group protonation may enhance the ability of DOM aromatic groups to act as π-acceptors through conversion of weakly electron-withdrawing carboxylate groups into more strongly electron-withdrawing carboxylic groups, effectively reducing the π-electron density in DOM substituted aromatic groups.[41] Provided the changes in cephalexin and DOM speciation shown here with pH variation itself, all these explanations were feasible in this study. Increased DOM carboxyl group protonation as well as reduced net negative charges on both DOM and cephalexin all potentially align with hydrophobic interactions, H-bonding, electrostatics and/or π-π interactions being the most likely mechanisms based strictly on observed pH trends.

Ionic strength and cation charge impact on cephalexin-DOM interactions

DOM fluorescence quenching in deionized ultrapure water, I = 0.10 M NaCl and I = 0.10 M $CaCl_2$ at pH = 7.0 occurred under all three conditions (**Figures 5** and **S5)**. Ryan-Weber models for observed quenching show cephalexin-DOM binding in deionized ultrapure water, I = 0.10 M NaCl and I = 0.10 M $CaCl_2$ had log K_c values of 6.00, 6.41 and 5.93, respectively (**Figure 5, Table S4**). These conditional equilibrium coefficient values were comparatively similar compared to the pH variation study, which showed several orders of magnitude difference across the pH treatments. This suggests that cephalexin-DOM binding was largely insensitive to changes in ionic strength and charge of cations in solution. The L_t values observed for varying ionic strength and background cation studies were also comparatively similar, with deionized ultrapure water, I = 0.10 M NaCl and I = 0.10 M $CaCl_2$ having L_t values of $2.41 \cdot 10^{-3}$, $2.16 \cdot 10^{-3}$ and $1.78 \cdot 10^{-3}$ M, respectively. It should be noted that the fluorescence of DOM itself

(i.e., without cephalexin present) was not influenced by interaction with Ca^{2+} as shown by the similarity in fluorescence intensity with no cephalexin present between differing ionic strength and background cation (**Figure S5**).

Despite the scarcity of these trends in the literature, similarity of natural organic matter binding characteristics with differing ionic strength and cation charge provides some insight into which mechanisms drive cephalexin-DOM interaction. An absence of Ca^{2+} impact on cephalexin-DOM binding indicated that cation bridging is not a contributing mechanism, as Ca^{2+} would enhance binding through interaction with the COO^- group of cephalexin and negatively charged COO^- groups on DOM.[47] The resulting COO-Ca^{2+}-OOC complex, formed by ternary complexation or electrostatic interaction, would be enhanced in the presence of Ca^{2+} if cation bridging were a factor, which was not observed here.[48] Insensitivity to ionic strength, as observed in this study, was previously related to hydrophobic interactions of the pharmaceutical propranolol with humic and fulvic acid natural organic matter fractions.[20] This interpretation, however, was inconsistent with the influence of ionic strength on the conformation of natural organic matter. As ionic strength increases natural organic matter becomes more structurally condensed through screening of negatively charged functional groups, effectively producing a less hydrated environment more favorable for hydrophobic interactions.[49] This effect also would make electrostatic interactions more favorable if electrostatic repulsion is dominant and less favorable in the instance of electrostatic attraction.[50] In addition to these considerations regarding DOM conformation, changes in ionic strength and background cation charge would have a competitive effect with cephalexin on DOM exchange sites if electrostatic interactions influenced binding. In the case of a DOM matrix where negative charges would prevail, the ability of positively charged amine moieties on cephalexin to associate with negatively charged

groups of DOM (e.g., carboxylate) would be hindered in the presence of a higher ionic strength solution where higher concentration of cations would engage in competition with cephalexin for exchange sites.[51] Alternatively, if cation bridging played a role in cephalexin-DOM binding, interaction would get stronger with increasing ionic strength in the presence of divalent cations, like Ca^{2+}.[48] Neither of these trends was observed for altering ionic strength, however, indicating that conformational changes that may be incurred by DOM and shorter-range ionic interactions do not impact cephalexin-DOM interaction. These points would indicate that hydrophobic, electrostatic and cation bridging interactions were likely not involved in cephalexin-DOM binding. They do, however, provide further evidence that π-π interactions play an important role in this process, as previous studies have suggested that π- π bonding may be insensitive to changes in ionic strength.[52,53]

Proposed mechanism of cephalexin-DOM interaction

Collective results from the pH, ionic strength and cation charge variation experiments can be used to identify the underlying cephalexin-DOM binding mechanism. The pH variation study showed cephalexin-DOM binding is stronger at lower pH values, indicating hydrophobic, H-bonding, electrostatic and/or π-π interactions as most probable mechanisms. Insensitivity of cephalexin-DOM interaction to changes in solution ionic strength and cation charge ruled out cation bridging, electrostatic and hydrophobic interactions, pointing toward π-π interactions and/or H-bonding as the probable modes of cephalexin-DOM binding. In addition to the binding response to these solution variables, the aromatic ring in cephalexin as well as the prevalence of aromatic and phenolic functional groups in the extracted biosolids DOM, as shown by ATR-FTIR, suggest that compatible molecular structures for π-π interaction are present. Moreover, π-π interaction fundamentally corresponds well with the quenching, and therefore involvement, of

DOM aromatic functional groups by cephalexin association. The molecular specificity of π-π interactions, which require the involvement of two aromatic functional groups, would also conceptually align with the 1:1 binding stoichiometry assumed for the Ryan-Weber model. H-bonding may also contribute to cephalexin-DOM interactions based on observed trends with solution chemistry. Cephalexin also contains functional groups capable of participating in H-bonding, such as carbonyls and N-containing groups. These groups have previously been attributed to H-bonding interactions between several antibiotics and natural organic matter [11]. The lack of spectroscopic evidence, as shown for DOM aromatic functional group involvement in π-π interactions, however, renders this mechanism less apparent.

Comparison between cephalexin and other pharmaceutical interactions with DOM

From a mechanistic perspective, the findings of π-π interactions serving as the dominant mechanism for cephalexin-DOM binding relate well to several previous studies in which antibiotic binding to natural organic matter is studied. For example, Christl et al. identified π-π bonding as a likely mechanism for tetracycline binding onto natural organic matter.[48] In another study, π-π interactions were proposed for the association of ciprofloxacin with humic and fulvic acids.[54] Along with this current study, these results highlight the significance of antibiotic aromatic functional groups in the association with natural organic matter fractions.

Cephalexin-DOM interaction quantitatively corresponds well with existing studies for organic contaminant-DOM interactions as well. Previous results showed the binding of the organoarsenic compound roxarsone to DOM yielded a log K_c of 5.06.[25] Another report showed carbamazepine binding to estuarine DOM had log K_c values from 3.10 to 5.23.[26] Our findings with pH = 7.0 synthetic wastewater as well as in DIW, I=0.10 M NaCl and I = 0.10 M $CaCl_2$ corresponded well with these previous reported values. At pH = 4.0 and 5.0 in synthetic

384 wastewater, however, our observed cephalexin complexation coefficients are appreciably higher.
385 This is possibly due to the conditions under which binding was studied, as experiments with
386 roxarsone were conducted at pH = 6.0 or in DOM solutions ranging from pH = 7.58-7.70 for
387 carbamazepine. It is also likely that differences in DOM composition and contaminant structure
388 played some role in the observed differences in log K_c values between our study and previous
389 reports. It should be noted that extent of interaction with DOM for these contaminants was
390 sufficient to influence photodegradation and adsorption processes.[55–57]

391 While the literature on cephalexin and β-lactam interaction with DOM and natural
392 organic matter is scarce, some previous studies with these compounds and organic biochar media
393 have been conducted to compare with the current study. Acelas et al. concluded through varying
394 solution pH that H-bonding through cephalexin carbonyl and carboxyl groups and electrostatic
395 interactions between positively charged amine groups on cephalexin and negative biochar groups
396 were the primary modes of adsorption.[58] π-π interactions were considered a likely contributor to
397 cephalexin adsorption as well.[58] This aligns with the results of our study, with π-π interactions
398 and H-bonding playing a role in cephalexin binding. Electrostatic interactions between the amine
399 group of cephalexin and negatively charged groups were not, however, likely to play a role in
400 our study as binding was insensitive to changes in ionic strength and cation charge. This
401 disparity between studies could be explained by the experimental pH ranges investigated, where
402 the previous study utilized values as low as pH = 2.0, where cephalexin speciation is expected to
403 be largely positive. The importance of π-π interactions in particular in the retention of another β-
404 lactam antibiotic (amoxicillin) by biochar was shown by another study.[59] How these results
405 translate to binding of other β-lactam antibiotics to DOM, and natural organic matter in general,
406 represents an area of potential future research.

Impact of DOM on cephalexin removal from solution by biochar and montmorillonite

Cephalexin adsorption behavior from a synthetic wastewater matrix by biochar and montmorillonite adsorbents at pH = 7.0 showed the influence of DOM on removal (**Figure 6a**). K_d values determined for 100 μg L^{-1} initial cephalexin concentration with biochar and clay adsorbents were 1.99 and 1.77 • 10^3 L kg^{-1}, respectively. These strong partitioning affinities are likely due to the mechanism of cephalexin adsorption on biochar and clay, both of which have been linked with high adsorption affinities.[17,58] In addition to the previously described π-π interaction, H-bonding and electrostatic mechanisms, cephalexin may become occluded in biochar pores.[19,58,60] Adsorption of cephalexin on smectite clays is primarily driven through electrostatic interactions and H-bonding to the clay surface through water bridging.[17] Additionally, adsorption of cephalexin and other β-lactam antibiotics was determined by X-ray diffraction to be limited to external clay particle surfaces, with interparticle clay pores not contributing to adsorption.[17] With the addition of 15 mg L^{-1} C Milorganite DOM, K_d values for biochar and montmorillonite were significantly reduced to 6.00 • 10^1 and 5.51 • 10^2 L kg^{-1}, respectively. This trend in observed partitioning behavior translated to significant differences in removal efficiency for both adsorbents as well. For biochar, a removal efficiency of 79.8% was observed without DOM and was reduced to 10.7% with DOM (**Figure 6b**). A less pronounced decrease was observed for montmorillonite, with 77.8% removal without DOM reduced to 52.3% in the presence of DOM. While both K_d values and removal efficiencies showed clear reduction of cephalexin adsorption in the presence of DOM, the extent to which reduction occurred differed between adsorbents, with adsorption onto biochar impacted more than onto montmorillonite. These results collectively showed the influence of DOM on cephalexin removal

429 from solution under environmentally relevant solution conditions and conditions in which
430 cephalexin-DOM interaction occurred.

431 Reduction of antibiotic adsorption onto environmental media (e.g., soil, clay, biochar) by
432 DOM is commonly observed and often attributed to antibiotic-DOM solution interactions
433 hindering adsorption.[16,17,61] Studies showing the impact of DOM on adsorption, however, often
434 do not demonstrate antibiotic-DOM association in solution. Here, we established that DOM
435 hinders cephalexin adsorption onto biochar and clay under conditions that favor formation of
436 solution cephalexin-DOM complexes (i.e., in a synthetic wastewater matrix at pH = 7.0),
437 providing evidence for the importance of cephalexin-DOM binding in the adsorptive removal of
438 cephalexin from wastewater. Cephalexin-DOM binding may not be the only process by which
439 DOM reduced cephalexin adsorption onto these adsorbents. It has been shown that competition
440 between DOM and solutes for sorption sites as well as DOM blocking of microporous structures
441 may also inhibit organic contaminant partitioning onto clay and biochar adsorbents.[13,62] As such,
442 it is probable that those influences played some role here as well. Provided the differences in
443 porosity and surface chemistry between biochar and montmorillonite and respective cephalexin
444 adsorption mechanisms, DOM blocking of pores and surface sites may have contributed to the
445 disparity in DOM impact on cephalexin adsorption behavior between adsorbents. Microporous
446 structures of biochar that serve as important sites for antibiotic adsorption may in particular be
447 more susceptible to blockage by DOM and also be less accessible to larger formed cephalexin-
448 DOM assemblies.[19] Differences in structural pore characteristics between biochar and clay could
449 make the effect of DOM less pronounced relative to biochar for clay from these perspectives.
450 This is particularly a relevant consideration given the adsorption of cephalexin to clay is on the
451 outer clay surfaces, rather than interlayer spaces.[17] This could allow surface sites accessible to

cephalexin to be accessible to larger cephalexin-DOM complexes as well on clay, which may allow for greater retention of cephalexin adsorption despite complexation. These results indicate that making pores inaccessible by DOM, either through complexation to cephalexin or through direct blocking of pores by DOM not bound to cephalexin, likely influences the reduction of cephalexin adsorption in the presence of DOM. Despite this evidence, the specific role(s) of contaminant-DOM interaction on adsorptive removal compared with pore and surface site blocking warrants further investigation in conjunction with solute interaction studies. Use of differing contaminants and adsorbent surface structures may be particularly informative when assessing these considerations.

Conclusions

This study developed a novel perspective on the interaction between DOM and cephalexin, a process between prevalent constituents in wastewater streams with implications for antibiotic fate in the environment. This interaction was studied in a synthetic wastewater matrix at pH = 4.0, 5.0, 7.0 and 8.0 as well as in DIW, I = 0.1 M NaCl and I = 0.1 M $CaCl_2$. Fluorescence EEMs show that the dominant protein-like fluorophores of activated biosolids-derived DOM interacted with cephalexin under all conditions studied except for synthetic wastewater at pH = 8.0. Binding was increasingly strong with decreasing pH while being insensitive to ionic strength and background cation charge. These trends, along with the observed trend of increasing DOM protonation with decreasing pH and direct quenching of aromatic fluorophores by cephalexin, suggest that π-π interactions play a key role in the association of cephalexin with DOM. It is possible that H-bonding of DOM with cephalexin carbonyl and N-containing functional groups contributes as well. Furthermore, the potential implications of cephalexin-DOM interaction was probed through a batch adsorption experiment. K_d values and removal efficiencies determined

for cephalexin adsorption onto montmorillonite and biochar under conditions at which cephalexin-DOM binding occurs demonstrated that DOM significantly impedes cephalexin removal, albeit to varying extent. These results collectively demonstrated that cephalexin-DOM interaction occurs, identified the mechanism by which it occurs, determined which solution chemistry variables influence interaction and provided an example of a treatment related process that could potentially be impacted by this interaction with implications for wastewater management. For example, these results suggest reduction in cephalexin removal performance by the presence of DOM may be lower for montmorillonite than biochar in adsorption-based treatment systems, providing some guidance into adsorbent selection for antibiotic removal based upon water chemistry. These findings may more broadly inform future efforts for developing strategies to predict and manage antibiotic fate in wastewater streams and environmental systems which receive them, such as treated municipal wastewater irrigated agricultural systems.

Conflicts of Interest

There are no conflicts of interest to declare.

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662 **Figure 1.** Fluorescence EEM of 2.5 mg C L^{-1} Milorganite-derived DOM in ultrapure water

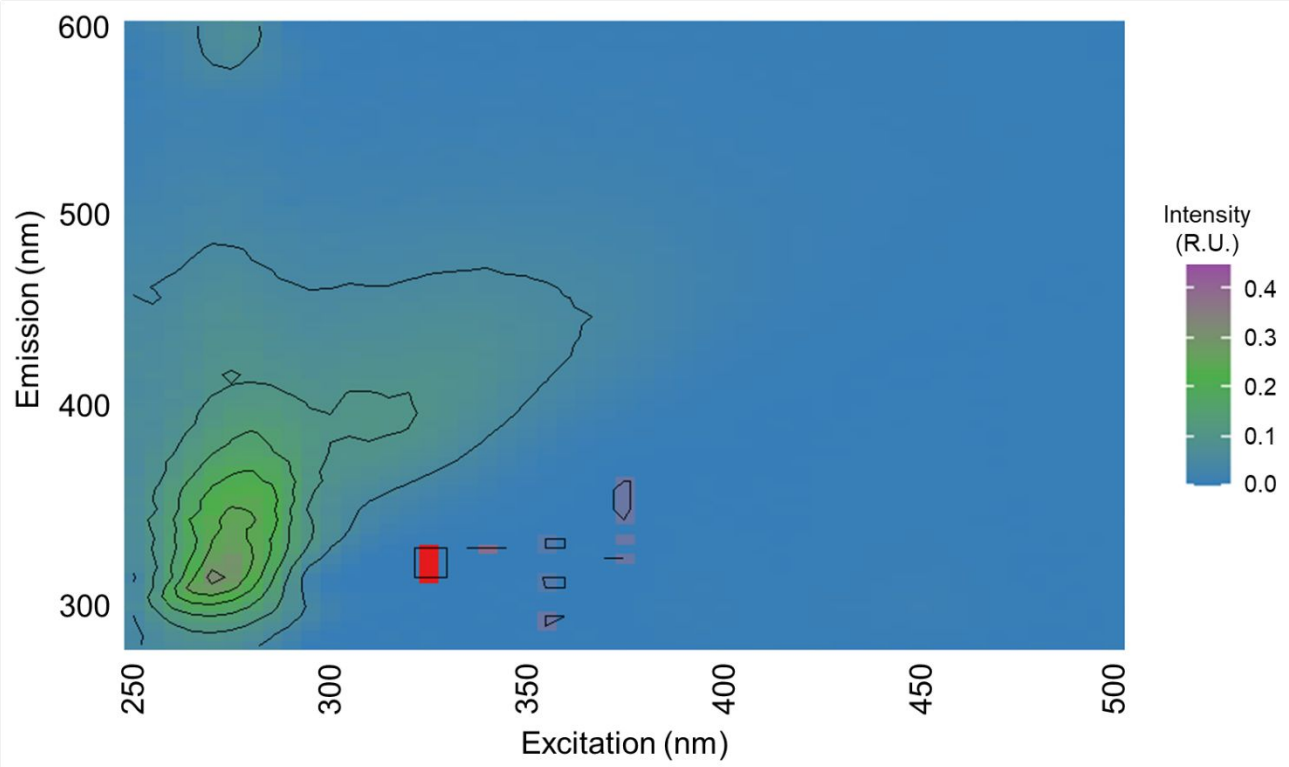

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Figure 2. ATR-FTIR spectra of biosolids-derived DOM (1500 mg L^{-1}) in synthetic wastewater solution at pH = 4.0, 5.0, 7.0 and 8.0.

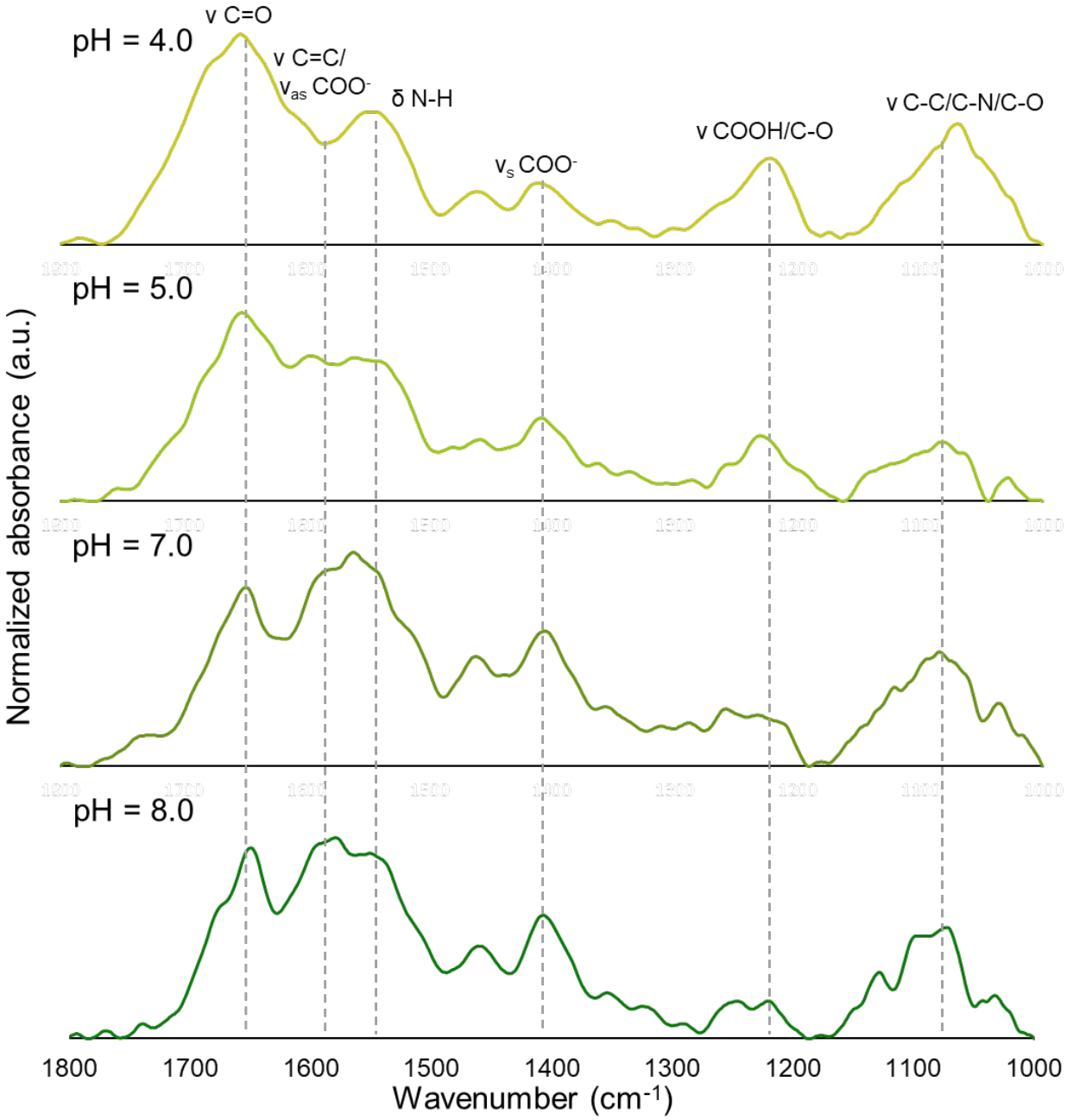
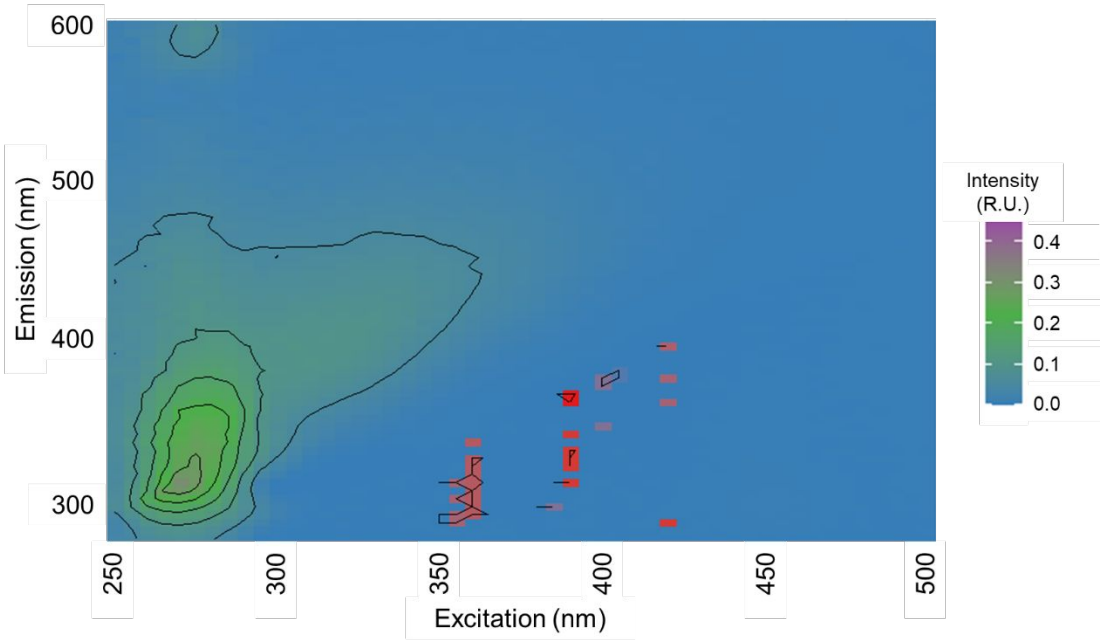
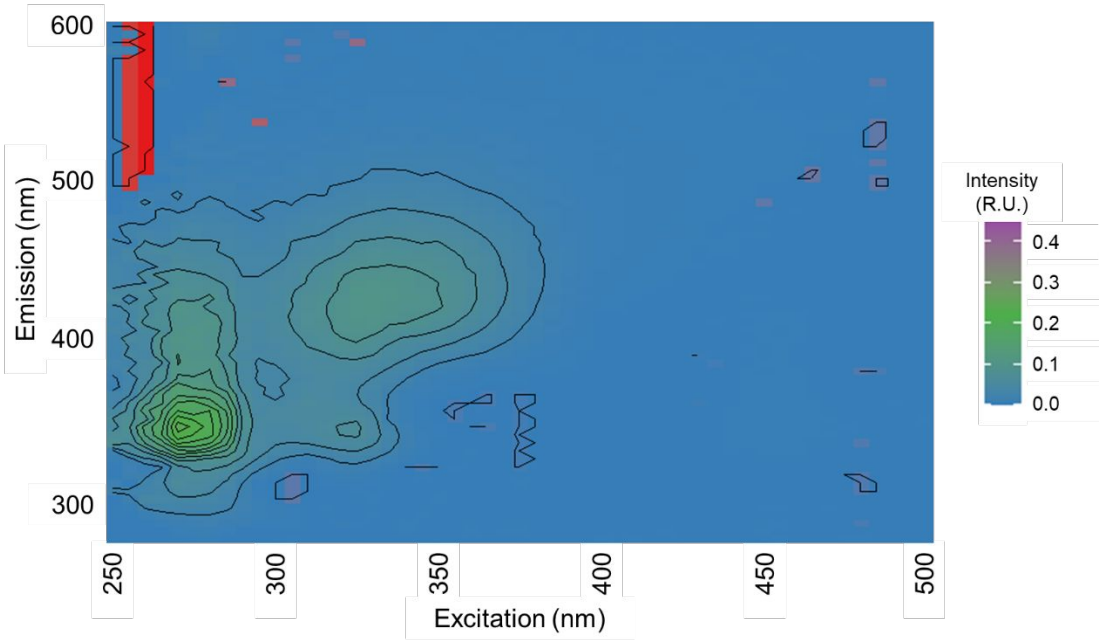


Figure 3. Example fluorescence EEMs of 2.5 mg C L^{-1} DOM in synthetic wastewater at pH = 5.0 with a) no cephalexin and b) with 5.74 x 10^{-4} M cephalexin.

a)



b)



696 **Figure 4.** Experimental fluorescence quenching results (points) and Ryan-Weber model fits
697 (dashed lines) for [DOM] = 2.5 mg C L^{-1} in synthetic wastewater at pH = 4.0 (red), 5.0 (yellow)
698 and 7.0 (blue). Error bars represent standard deviations of experimental replicates (n = 3).

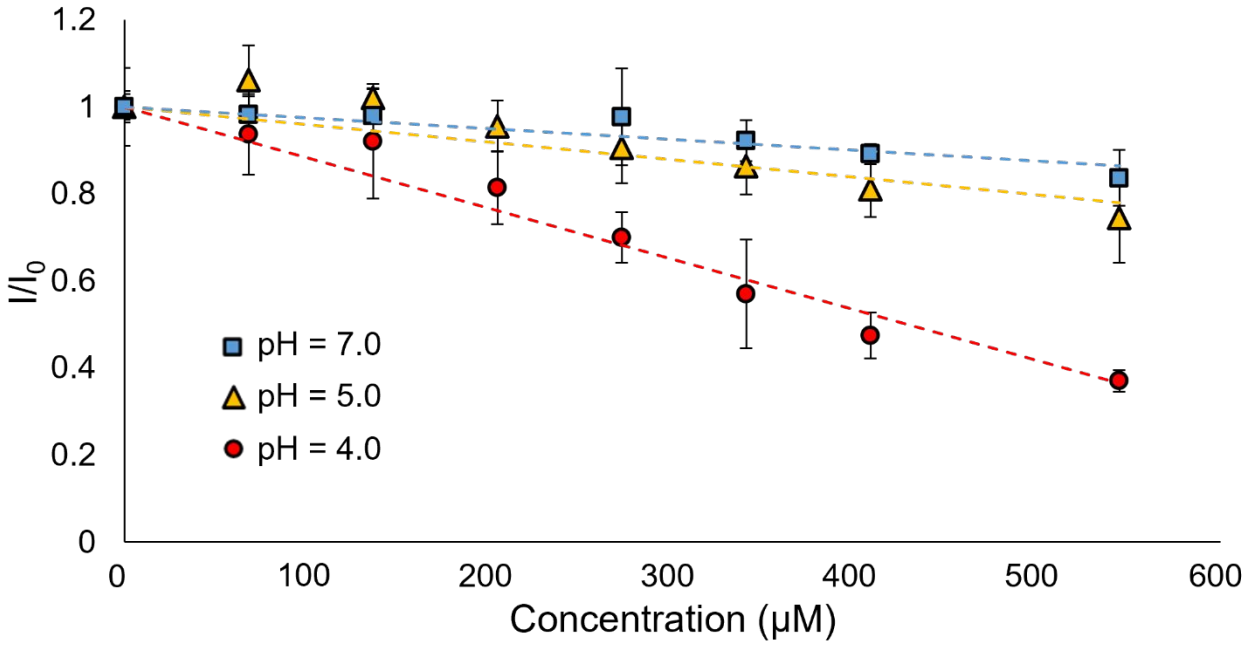


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Figure 5. Experimental fluorescence quenching results (points) and Ryan-Weber model fits (dashed lines) for [DOM] = 2.5 mg C L^{-1} in ultrapure water (green), I = 0.1 M NaCl (orange) and I = 0.1 M $CaCl_2$ (brown). Error bars represent standard deviations of experimental replicates (n = 3).

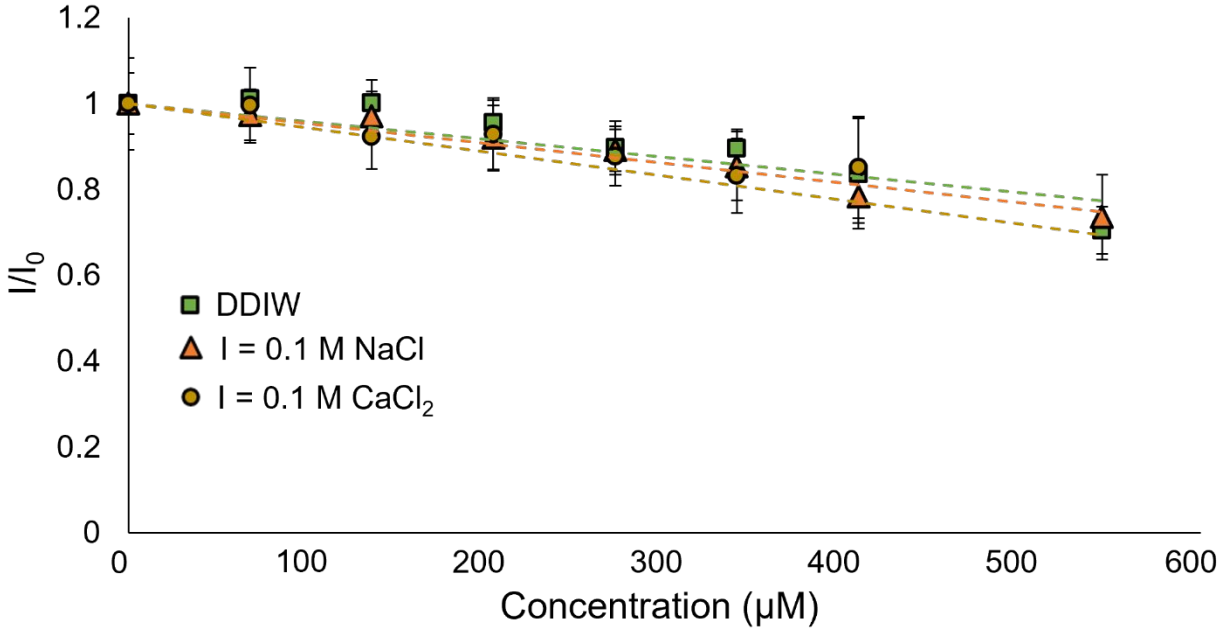
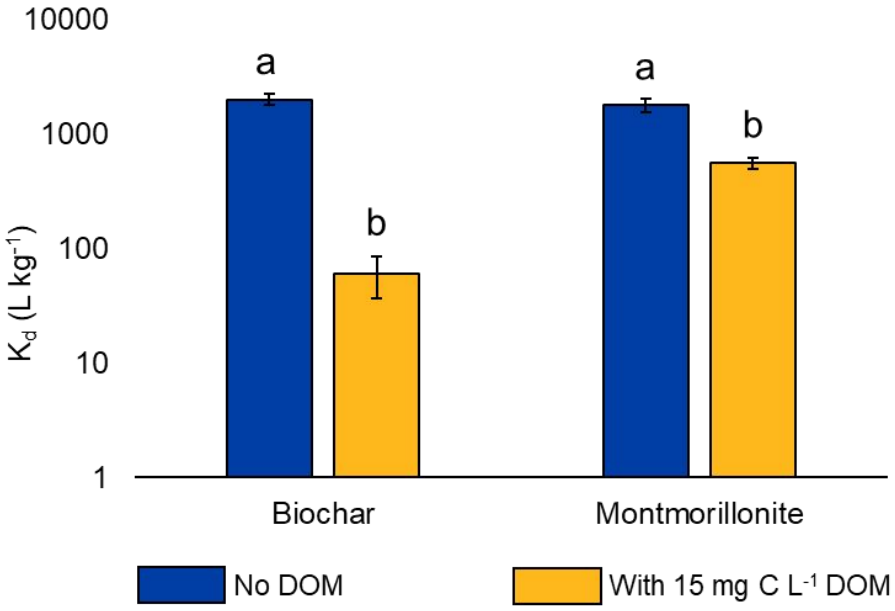


Figure 6. a) K_d values and b) removal efficiencies for cephalexin adsorption onto biochar and montmorillonite determined at 100 µg L^{-1} initial cephalexin concentration in synthetic wastewater at pH = 7.0 with and without 15 mg L^{-1} C DOM. Error bars denote standard deviation of experimental replicates (n = 3). Letters denote statistical significance between treatments with and without DOM for respective adsorbents as determined by Student's t-test ($p < 0.05$).

a)



b)